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CARACTERIZAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS DERIVADAS DO TECIDO ADIPOSO E SEU POTENCIAL DE DIFERENCIAÇÃO

Santa Maria, RS
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Tese apresentada ao Curso de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária.**

Orientador: Prof. Paulo Bayard Dias Gonçalves

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RESUMO

DESENVOLVIMENTO E CARACTERIZAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS DERIVADAS DO TECIDO ADIPOSO E SEU POTENCIAL DE DIFERENCIAÇÃO

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Células-tronco mesenquimais têm demonstrado significativo potencial para aplicação terapêutica devido ao seu fácil isolamento, baixa imunogenicidade, ausência das implicações éticas e sua ampla plasticidade. Essas células estão nos mais diversos tecidos, destacando-se o tecido adiposo devido á sua ampla distribuição no organismo, conveniente obtenção e o considerável número de células-tronco mesenquimais multipotentes que podem ser isoladas desse tecido. Assim sendo, no presente estudo, células-tronco mesenquimais derivadas do tecido adiposo (AT-MSCs) foram isoladas do tecido adiposo localizado nas regiões próximas ao omento e testículos de camundongos BALB/c. Durante a manutenção e expansão das AT-MSCs *in vitro*, elas foram caracterizadas quanto à presença de marcadores antigênicos de superfície e potencial de diferenciação nas linhagens osteogênica, condrogênica e adipogênica. AT-MSCs de ambas as fontes expressaram os marcadores mesenquimais de superfície, CD73 e CD105, assim como foram negativas para o marcador de linhagens hematopoiéticas, CD45. Quanto ao potencial de diferenciação, os cultivos provenientes das duas origens de tecido adiposo apresentaram capacidade de diferenciar nas três linhagens acima citadas. Porém, foram observadas discretas diferenças tanto nos padrões de expressão dos marcadores mesenquimais de superfície quanto nos potenciais de diferenciação entre as AT-MSCs provenientes dos diferentes locais de deposição de gordura. Além disso, as AT-MSCs isoladas do tecido adiposo depositado em contato com o omento quando cultivadas com meios de diferenciação, contendo ácido retinóico e meio condicionado testicular demonstraram expressão do gene *Gdnf* o qual é reconhecidamente expresso pelas células de Sertoli. Portanto, os resultados obtidos demonstram que conforme a origem do tecido adiposo as AT-MSCs possuem diferentes características relacionadas aos marcadores de superfície assim como aos potenciais de diferenciação.

Palavras-chave: Ácido retinóico. Células multipotentes da fração estromal vascular. Diferenciação *in vitro*. Gene marcador. Marcadores antigênicos de superfície. Meio condicionado.

ABSTRACT

DEVELOPMENT, CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

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ADVISER: Prof. Paulo Bayard Dias Gonçalves

Mesenchymal stem cells (MSCs) have demonstrated significant potential for clinical use due to their convenient isolation, lack of significant immunogenicity, lack of ethical controversy and their potential to differentiate into tissue-specific cell types. MSCs reside in almost all tissues including the adipose tissue. Adipose tissue has main advantages as wide distribution in the organism, suitable isolation and considerable amount of resident multipotent stem cells. Therefore, in this study, adipose tissue-derived mesenchymal stem cells (AT-MSCs) were isolated from BALB/c mice omentum and epididymis fat pats. During AT-MSCs maintenance and expansion *in vitro*, they were characterized for the expression of antigenic surface markers and for osteogenic, chondrogenic, and adipogenic differentiation potential. AT-MSCs form both sources expressed mesenchymal surface markers, CD73, and CD105 and were negative for a hematopoietic marker, CD45. The cultures derived from both adipose tissues differentiated into all three lineages. However, differences were observed in mesenchymal surface marker expression profiles as well as in the differentiation potential of AT-MSCs from different fat sources. Furthermore, AT-MSCs isolated from omentum fat depot were cultured with differentiation medium containing retinoic acid and testicular cell conditioned medium. After treatment periods, AT-MSCs showed *Gdnf* gene expression, this gene is a marker for Sertoli cells. The results showed that AT-MSCs from distinct fat depots have different characteristics related to stem cell surface marker expression profiles and differentiation potential.

Keywords: Antigenic surface markers. Conditioned medium. Gene marker. *In vitro* differentiation. Multipotent stromal cells. Retinoic acid.

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1 INTRODUÇÃO

As células-tronco adultas possuem plasticidade mais ampla do que previamente suposto, ultrapassando as barreiras restritas às linhagens do tecido de origem (WAGERS; WEISSMAN, 2004; ZUK et al., 2002). Os tecidos dos mamíferos pós-nascimento possuem pequenas populações dessas células com capacidade de auto-renovar, proliferar e diferenciar em células especializadas, promovendo renovação e reparo do tecido (PISCAGLIA, 2008). O tecido adiposo que está amplamente distribuído no organismo é uma fonte de células-tronco adultas multipotentes em significativa quantidade e de fácil obtenção (FRASER et al., 2007; MITCHELL et al., 2006). Esse tecido é dividido em duas diferentes frações, os adipócitos maduros e a fração estromal vascular onde estão as células-tronco mesenquimais (MSCs). As células-tronco mesenquimais derivadas do tecido adiposo (AT-MSCs) possuem ampla plasticidade, padrão complexo de expressão de moléculas marcadoras de superfície (CDs), além de secretar fatores relacionados ás respostas imune e inflamatória (BUNNELL et al., 2008; MOSNA et al., 2010; STREM et al., 2005; ZUK et al., 2002).

Quando cultivadas em meios contendo fatores de indução de diferenciação as MSCs podem transdiferenciar *in vitro* em diversas linhagens celulares (DA SILVA et al., 2013; LACHAM-KAPLAN et al., 2006; MORTAZAVI; MOHAMMADI, 2013; SCHÄFLER; BÜCHLEER, 2007; ZUK et al., 2002). Além disso, o meio condicionado proveniente do sobrenadante de um cultivo celular contém diversos fatores solúveis que poderão auxiliar na diferenciação das MSCs em linhagens celulares relacionadas com o cultivo (DA SILVA et al., 2013; LACHAM-KAPLAN et al., 2006; MORTAZAVI; MOHAMMADI, 2013). Portanto, os meios de diferenciação contendo os fatores de indução assim como o meio condicionado são abordagens empregadas para promover a diferenciação das AT-MSCs em diversas linhagens celulares (DRUSENHEIMER et al., 2006; HUANG et al., 2010; NAYERNIA et al., 2006; ZHANG et al., 2014). Sendo que o potencial de diferenciação de MSCs em células germinativas masculinas já foi abordado em alguns trabalhos (DRUSENHEIMER et al., 2006; HUANG et al., 2010; ZHANG et al., 2014). Entretanto, o potencial de diferenciação de AT-MSCs em células germinativas masculinas necessita ser elucidado. Assim como, necessita-se esclarecer o potencial de ação dos meios suplementado com ácido retinóico e condicionado testicular no processo de diferenciação das AT-MSCs em células germinativas masculinas.

Portanto, os objetivos deste estudo foram desenvolver e caracterizar as AT-MSCs isoladas de diferentes origens de tecido adiposo, além de verificar seu potencial de diferenciar

em linhagens celulares relacionadas às células germinativas masculinas. Para isso, primeiramente, foram estabelecidos cultivos das frações estromal vascular obtidas de dois diferentes depósitos de gordura em camundongos BALB/c. A caracterização das AT-MSCs foi realizada pela presença de marcadores de superfície por citometria de fluxo e pela capacidade de diferenciação adipogênica, osteogênica e condrogênica. Ademais, após os tratamentos com ácido retinóico e/ou meio condicionado proveniente do cultivo de células do testículo de camundongos da mesma espécie, o possível potencial de diferenciação das AT-MSCs em linhagens celulares relacionadas às células germinativas masculinas foi verificado por meio da expressão de genes marcadores específicoa (CHEN & LIU, 2015; HOU et al., 2014; IKAMI et al., 2015; MENG et al., 2000).

2 REVISÃO BIBLIOGRÁFICA

2.1 CÉLULAS-TRONCO

As células-tronco são um tipo especial de célula não especializada com capacidade de auto-renovar, proliferar e diferenciar em diversas linhagens celulares (BLAU et al., 2001; VERFAILLIE, 2002). A função fundamental dessas células *in vivo* é participar nos processos de renovação, reparo e regeneração dos tecidos (BLAU et al., 2001). Células-tronco são classificadas quanto a sua origem e plasticidade sendo que a classificação mais empregada é em embrionárias ou adultas. As células-tronco embrionárias são pluripotentes, ou seja, possuem ampla capacidade de diferenciação, podendo diferenciar-se em todos os tipos celulares, e encontram-se na massa celular interna do blastocisto e na prega genital do feto de 5-10 semanas. Já as células-tronco adultas (somáticas; multi ou unipotentes) possuem capacidade de diferenciação limitada e residem nos tecidos dos mamíferos como medula óssea, cordão umbilical, polpa dentária dentre outros (BLUM et al., 2009; PISCAGLIA, 2008; SU et al., 2011).

Os primeiros estudos envolvendo células-tronco foram realizados utilizando as células embrionárias, objetivando empregá-las na terapia de diferentes patologias (KAJI;e LEIDEN, 2001) como facilitadoras do reparo de tecidos degenerados ou lesionados, porém a aplicação terapêutica dessas células passou a apresentar diversos problemas, como rejeição devido à incompatibilidade imunológica, perda da função das células infundidas, amplo potencial de formação de teratomas, além das questões éticas e legais envolvendo células-tronco embrionárias (BLAU et al., 2001; BLUM et al., 2009; PISCAGLIA, 2008; SU et al., 2011). Sabe-se, desde os anos 60, que alguns tecidos de um organismo adulto se regeneram constantemente, isso acontece com a pele, com as paredes intestinais e principalmente com o sangue, que têm suas células destruídas e renovadas, em um complexo processo de proliferação e diferenciação celular finamente regulado. Consequentemente, pesquisadores isolaram células-tronco adultas de diversos tecidos, como medula óssea, tecido adiposo, cordão umbilical, pele, polpa dentária, entre outros, tornando-as uma alternativa ao uso das células embrionárias, já que minimizam a possibilidade de formação de tumores e não envolvem questões éticas e legais (BLAU et al., 2001; PITTENGER et al., 1999).

2.1.1 Células-tronco mesenquimais

Células-tronco mesenquimais (MSCs) são células multipotentes que podem originar, *in vitro,* linhagens mesenquimias e não mesenquimais e são as céluas-tronco adultas mais plásticas conhecidas até o momento (DA SILVA et al., 2006; ZUK et al., 2002). MSCs possuem ampla distribuição natural no organismo dos mamíferos pós-nascimento, sendo preferencialmente encontradas nos tecidos conjuntivos e foram isoladas com sucesso de vários tecidos como medula óssea (PITTENGER et al., 1999), músculo esquelético (JANKOWSKI et al., 2002), dentes decíduos (MIURA et al., 2003), tecido adiposo (ZUK et al., 2002) e paredes dos vasos sanguíneos (DA SILVA et al., 2006).

MSCs apresentam uma variedade de receptores e marcadores antigênicos de membrana, esses facilitam sua identificação, caracterização e isolamento. Uma variedade de marcadores de superfície (CDs) estão presentes nas MSCs: CD29, CD34, CD44, CD105, CD73, CD90, sendo que a checagem da expressão desses marcadores é amplamente utilizada para identificação das MSCs (MARCUS et al., 2008; DUBEY et al., 2014; JIANG et al., 2002; MOSNA et al., 2010; PITTENGER et al., 1999; ZUK et al., 2002). Essas células também secretam uma ampla variedade de fatores de crescimento e citocinas que possuem função mediadora nas respostas inflamatória, imune e na migração celular (CHAMBERLAIN et al., 2007; MOSNA et al., 2010). Além disso, outra característica importante das MSCs, no contexto de terapia celular, está no status imunológico privilegiado apresentado por essas células, o qual possibilita aplicação de células alogênicas com pouca implicação de resposta imune indesejada. Acredita-se que essa característica está associada com a baixa expressão dos antígenos do complexo de histocompatibilidade (MHC) (CHAMBERLAIN et al., 2007; MOSNA et al., 2010).

A população de células-tronco mesenquimais derivadas da medula óssea (BM-MSCs) é a mais pesquisada, principalmente para aplicação na terapia celular regenerativa, desde sua descoberta nos anos 70 por Friedenstein (CHAMBERLAIN et al., 2007; PHINNEY & PROCKOP, 2007). O desenvolvimento de protocolos para isolar, purificar e cultivar BM-MSC já foram descritos detalhadamente em inúmeras publicações (MOSNA et al., 2010; PITTENGER et al., 1999; REYES et al., 2001). Células-tronco mesenquimais derivadas do tecido adiposo (AT-MSCs) representam uma alternativa atrativa às derivadas da medula óssea devido sua abundância, acessibilidade, ampla plasticidade e ausência de imunogenicidade (GIMBLE et al., 2007). Em adultos, estima-se que a fração de MSCs proveniente da medula óssea é de 1:50.000 - 1:1 milhão do total de células nucleadas. Essas são obtidas a partir de

um volume limitado que pode ser puncionado de forma segura por doador, fazendo com que seja necessária significativa expansão *in vitro* (MUSCHLER et al., 2001). Em contraste, a frequência de MSCs no tecido adiposo é estimada numa faixa entre 1:30-1:100 pelo total de células nucleadas (FRASER et al., 2007; MITCHELL et al., 2006). Além disso, quantidade significativa de tecido adiposo pode ser retirado do paciente sem prejuízo para saúde desse, como por exemplo, os procedimentos de lipoaspiração. As BM-MSCs e as AT-MSCs possuem várias características em comum, além da multipotência, como a habilidade de proliferar indefinidamente em cultivo e a presença de marcadores característicos na superfície (PITTENGER et al., 1999). Porém, as AT-MSCs apresentam vantagens como relativa abundância e facilidade de obtenção quando comparadas as BM-MSCs. Isso torna as AT-MSCs uma excelente fonte alternativa de célula-tronco (FRASER et al., 2007; GIMBLE et al., 2007; MITCHELL et al., 2006).

2.2 TECIDO ADIPOSO

O tecido adiposo é originado durante o desenvolvimento embrionário a partir da mesoderme, contém um estroma de suporte o qual é facilmente isolado e representa uma fonte de células-tronco de fácil obtenção. Em um organismo adulto, as fontes de tecido adiposo são diversas, podendo ser coletado da região subcutânea, da região visceral, das regiões próximas e em contato com os órgãos e gônadas (BUNNELL et al., 2008; STREM et al., 2005). Acreditava-se que o tecido adiposo estava simplesmente envolvido no armazenamento de lipídios, porém atualmente, já é reconhecido por possuir alta atividade endócrina metabólica, pois secreta citocinas, (adipocinas) como leptina, adiponectina e interleucina 6 (IL-6) (KIM et al., 2000). A localização no organismo do depósito de gordura pode influenciar nas características das AT-MSCs, alguns estudos demonstraram que células-tronco adiposas provenientes do subcutâneo são mais adipogênicas que as extraídas do omento (PARK et al., 2012; TCHKONIA et al., 2002). Além disso, podem variar os padrões dos fatores secretados e dos genes expressos entre as fontes de gordura (ATZMON et al., 2002; MAJKA et al., 2011).

Constantemente, durante toda a vida dos mamíferos o tecido adiposo se expande e reduz demonstrando capacidade de auto-renovação, e de diferenciação, além de metabólica, das células presentes nesse tecido (STREM et al., 2005). Além disso, o tecido adiposo é um tecido complexo composto por: adipócitos maduros, pré-adipócitos, fibroblastos, células da musculatura lisa vascular, células endoteliais, macrófagos/monócitos residentes

(SCHÄFFLER & BÜCHLER, 2007). Esse tecido pode ser basicamente dividido em duas diferentes frações, adipócitos maduros e a fração estromal vascular que possui composição celular heterogênea e onde se encontram as células-tronco multipotentes derivadas do tecido adiposo (PRUNET-MARCASSUS et al., 2006). As células que compõem a fração estromal vascular podem ser enzimaticamente isoladas, separadas dos adipócitos por centrifugação e mantidas em cultivo (BUNNELL et al., 2008).

Estudos indicando que as células-tronco adultas podem diferenciar para além dos limites restritos às linhagens do tecido, sugerindo ampla plasticidade (WAGERS; WEISSMAN, 2004), fez com que surgissem diversos trabalhos pesquisando o potencial de transdiferenciação das MSCs (JIANG et al., 2002; PITTENGER et al., 1999; ZUK et al., 2002). A habilidade de diferenciação das AT-MSCs em linhagens celulares mesenquimais clássicas como: adipócitos, condrócitos, osteócitos e miócitos (BUNNELL et al., 2008; STREM et al., 2005; ZUK et al., 2002), além da capacidade de diferenciação em linhagens não mesenquimais como tipos celulares esquelético e neuronal demonstra o potencial plástico dessas células, bem como sua potencial aplicação futura nas terapias celulares (ZUK et al., 2002).

2.3 DIFERENCIAÇÃO *IN VITRO*

In vivo, o microambiente ou nicho em que a célula-tronco adulta se encontra contém diversos e complexos componentes que sinalizam o destino dessa célula (WATT; HOGAN, 2000). O nicho é composto por parte das células do tecido e de substâncias extracelulares que abrigam as células-tronco e controlam sua auto-renovação ou diferenciação. O destino é regulado pela interação entre sinais endógenos e estímulos do microambiente, esse processo fino e complexamente regulado controla a manutenção e a diferenciação das células-tronco nos tecidos adultos (STEWART; STEWART, 2011; WATT; HOGAN, 2000). As condições de cultivo tentam reproduzir, *in vitro*, o papel do microambiente e assim direcionar as células-tronco a auto renovar, proliferar ou diferenciar (GEENS et al., 2011; LACHAM-KAPLAN et al., 2006; MANNELO; TONTI, 2007). Portanto, inúmeros estudos estão sendo realizados para o desenvolvimento de meios de cultivo suplementados com diversas substâncias solúveis como fatores de crescimento, hormônios, proteínas, vitaminas, aminoácidos, dentre outros visando estimular *in vitro* a diferenciação para um determinado tipo celular (SCHÄFLER; BÜCHLEER, 2007; ZUK et al., 2002).

2.3.1 Meio Condicionado

O meio condicionado é obtido a partir do sobrenadante do cultivo de células e vem sendo empregado como um importante aliado para diferenciação *in vitro* (DA SILVA et al., 2013; LACHAM-KAPLAN et al., 2006; MORTAZAVI; MOHAMMADI, 2013). Sendo que o meio condicionado já foi utilizado para a diferenciação condrogênica, miogênica e neural de BM-SCs e AT-MSCs (DA SILVA et al., 2013; HAN et al., 2014; STERN-STRAETER et al., 2013). O meio condicionado contém diversos fatores solúveis que podem auxiliar na diferenciação em linhagens celulares relacionadas com o cultivo celular do qual foi obtido. O meio condicionado proveniente do cultivo de células do testículo é uma fonte de diversos fatores solúveis desconhecidos e alguns já revelados como a proteína morfogenética óssea 4 (BMP4), fator inibidor da leucemia (LIF), fator de crescimento fibroblástico básico (bFGF) e o fator 9 de crescimento e diferenciação (GDF-9), além de hormônios como a testosterona, os quais são necessários para o desenvolvimento e proliferação das células germinativas masculinas (CREEMERS et al., 2002; HULEIHEL; LUNENFELD, 2004; PELLEGRINI et al., 2003; TAKABAYASHI et al., 2001). Além dos meios de cultivo suplementado e condicionado outros métodos como feedlayers, cocultivo, scaffolds, dentre outros, vêm sendo empregados visando tornar as condições *in vitro* mais próximas da *in vivo*. Portanto, todas essas abordagens utilizam análogos dos componentes celulares e da matriz extracelular do nicho para potencializar os processos de proliferação, maturação e diferenciação das células-tronco (CHENG et al., 2009; FLYNN et al., 2006; MANNELLO; TONTI, 2007; SOUSA et al., 2002).

2.3.2 Potencial de diferenciação em linhagens germinativas masculinas

A obtenção de linhagens celulares germinativas a partir de células-tronco vem sendo alvo de pesquisas que visam alcançar uma alternativa para os tratamentos de infertilidade (MEHRABANL et al., 2015; ZHANG et al., 2014). Para tanto, necessita-se além de uma fonte adequada de células-tronco, as condições apropriadas de diferenciação. Algumas pesquisas se valeram da pluripotência das células-tronco embrionárias e obtiveram a derivação dessas em células germinativas primordiais, epermatozóides e oócitos (CHEN et al., 2007; CLARK et al., 2004; GEIJSEN et al., 2004; HUBNER et al., 2003; NAYERNIA et al., 2006; TOYOOKA et al., 2003). Assim como, MSCs derivadas da medula óssea e do cordão umbilical, após serem submetidas a protocolos de diferenciação contendo ácido

retinóico e esse podendo estar combinado com meio condicionado proveniente do cultivo de células do testículo, promoveram a diferenciação das MSCs em possíveis células germinativas primordiais masculinas (DRUSENHEIMER et al., 2006; HUANG et al., 2010; ZHANG et al., 2014).

2.4 ESPERMATOGÊNESE

As células germinativas masculinas passam por um processo complexo, denominado espermatogênese, o qual ocorre constantemente em machos e envolve múltiplas vias de proliferação e alguns passos de diferenciação celular. Em 1956, Oakerg descreveu a cinética da produção de espermatozoides em roedores, acreditando que a diferenciação de células germinativas requer uma população de células-tronco. Nos testículos de camundongos adultos existe uma população de células-tronco espermatogoniais adultas (SSCs), essas possuem a função de auto-renovação e, consequentemente manutenção e contínua produção de espermatozoides ao longo da vida do macho (BRINSTER; ZIMMERMANN, 1994). Sendo que nos diferentes estádios das células germinativas masculinas, diversos marcadores moleculares foram identificados. Destacando-se na célula germinativa primordial (CGP) os marcadores de célula germinativa precoce como os genes *Fragillis, Stella* e *Vasa* e os genes marcadores das SSCs, *Dazl* e *Stra8* (HOU et al., 2014). Além desses, um fator extremamente importante para o desenvolvimento e manutenção das SSCs é o fator neurotrófico derivado das células gliais (GDNF) o qual é liberado pelas células de Sertoli no nicho testicular. GDNF é um fator parácrino que promove a manutenção e auto-renovação das SSCs, sendo que o gene *Gdnf* é amplamente expresso nas células de Sertoli (CHEN & LIU, 2015; IKAMI et al., 2015; MENG et al., 2000).

Na espermatogênese também estão envolvidas diversas substâncias como hormônios, vitaminas, fatores de crescimento, citocinas e aminoácidos (TESARIK et al., 1998). O ácido retinóico (AR), um derivado ativo da vitamina A ou retinol, influencia na diferenciação germinativa sendo essencial na transição para a etapa de meiose, tanto nas células germinais masculinas como nas femininas. Receptores para ácido retinóico são expressos nas células germinativas e nas de Sertoli as quais podem ser estimuladas por esse ácido. O AR age dentro do núcleo sendo reconhecido por duas classes diferentes de receptores. Ambas as classes (RARs e AXRs) consistem em três tipos de receptores, α, β, e γ codificados por diferentes genes e que transmitem os sinais mediados pela ligação direta ao AR (ROSSI & DOLCI, 2013). Em roedores, retinóides estão envolvidos na regulação das funções testiculares os

quais parecem ser necessários para a espermatogênese, no desenvolvimento dos espermatócitos nos estágios precoces da meiose (HUANG et al., 2010; NAYERNIA et al., 2006; ROOIJ, 2001; SUGIMOTO, NABESHIMA; YOSHIDA, 2012). Pesquisas demonstraram que o AR estimula a expressão do gene *Stra8*, fundamental no processo de espermatogênese, induzindo a transição da espermatogônia indiferenciada pra espermatogônia diferenciada (ROSSI & DOLCI, 2013; ZHOU et al., 2008). Portanto, de maneira geral, o AR atua durante o processo de espermatogênese através da ação direta nas espermatogônias e indiretamente por meio de mudanças no padrão de expressão dos fatores parácrinos como o GDNF secretado pelas células de Sertoli (ROSSI & DOLCI, 2013).

Há um crescente interesse em aplicar as MSCs na medicina regenerativa, inclusive na terapia dos casos de infertilidade, portanto, devido ás suas diversas fontes, expansão prolongada *in vitro* e multipotência faz com que muitos estudos e esforços sejam feitos para isolar, caracterizar, expandir e melhor compreender as AT-MSCs, além de estimular sua plasticidade. Consolidando a aplicação futura dessas células na terapia celular e na engenharia de tecidos (GAUSTAD et al., 2004; PHINNEY; PROCKOP, 2007; MEHRABANI et al., 2015; MOSNA et al., 2010; ZHANG et al., 2014; ZUK et al., 2001).

3 CAPÍTULO I – EVALUATION AND COMPARISON OF ADIPOSE TISSUE-DERIVED STROMAL STEM CELLS FROM TWO DIFFERENT FAT DEPOTS

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Abstract

Adipose tissue is widely distributed within a mammals' organism, and previous studies have demonstrated that according to the fat pad location, the adipose tissue is composed of different cell subsets with unique characteristics. This study aimed to elucidate the basic stem cell surface markers and differentiation potential of adipose tissue-derived cells from the stromal vascular fraction isolated from the omentum and epididymis fat depots. These distinct adipose sources provided stromal cells which showed differences in the expression profiles of stem cell surface markers, CD105, CD73, and CD45, and differences in the potential to differentiate into adipogenic, oesteogenic, and chondrogenic mesodermal lineages. Although, omentum and epididymis fat pads provided stromal-stem cells exhibiting distinct characteristics, both fat depots represent possible sources of multipotent stem cell for further studies.

Keywords: Adipose-derived stromal cell. Antigenic surface markers. Differentiation potential. Immunophenotype. Site-specificity. Stromal vascular fraction.

Introduction

Adipose tissue is an organ of great heterogeneity and plasticity. Historically, it was believed to be involved only in lipid storage; however, adipose tissue has high metabolic endocrine activity and, secretes adipokines such as leptin, adiponectin, and interleukin 6 (IL-6) (KIM et al., 2000). Animals have diverse fat sources, which are divided into subcutaneous and internal fat depots (BUNNELL et al., 2008; STREM et al., 2005). According to the fat pad location, adipose tissue shows different metabolic properties, functions, genes expression profiles, antigenic features, and differentiation potential (ATZMON et al., 2002; MAJKA et al., 2011; TCHKONIA et al., 2002).

The cellular complexity of adipose tissue is divided in two different cell fractions: mature adipocytes and the stromal-vascular fraction (SVF). The SVF is highly heterogeneous,

containing populations of fibroblasts, endothelial cells, vascular smooth muscle cells, monocytes, hematopoietic cells, and somatic stem cells (PRUNET-MARCASSUS et al., 2006; SCHÄFFLER & BÜCHLER, 2007). This fraction can be enzymatically isolated, and separated from adipocytes by centrifugation, and maintained in culture (BUNNELL et al., 2008). Although SVF culture has classically been used to investigate preadipocyte differentiation into mature adipocytes, recent studies have shown that adipose tissue-derived cells from the SVF (ASVF) display broad differentiation potential, with the ability to develop into osteogenic, chondrogenic, myogenic, neurogenic, and cardiogenic lineages (PRUNET-MARCASSUS et al., 2006).

ASFVs expand manyfold reaching high passage numbers and, retaining growth capacity and differentiation potential (ZHU et al., 2008). However, the cellular composition and expression levels of surface markers vary among passages, particularly between freshly isolated cells and those at later passages. Previous studies have demonstrated that during culture, serial passages of adipose tissue-derived mesenchymal stem cells (AT-MSCs) show a progressive increase in mesenchymal markers (CD13, CD29, CD44, CD73, CD90, and CD105) (KARP & TEO, 2009; MOSNA et al., 2010).

The fat depot location influences AT-MSCs characteristics, and studies have demonstrated that subcutaneous adipose tissue is more adipogenic than that from the omentum (PARK et al., 2012; TCHKONIA et al., 2002), and inguinal adipose tissue appears to be the most plastic adipose tissue (PRUNET-MARCASSUS et al., 2006). Furthermore, gene expression profiles and secreted substances can vary with fat sources (ATZMON et al., 2002; MAJKA et al., 2011). Previous studies have demonstrated that AT-MSCs have great potential for self-renewal and plasticity that is similar to bone marrow-derived mesenchymal stem cells (BM-MSCS) (BUNNELL et al., 2008; STREM et al., 2005; ZUK et al., 2002). However, AT-MSCs are more abundant and less difficult to obtain than BM-MSCs (FRASER et al., 2007; GIMBLE et al., 2007; MITCHELL et al., 2006). Therefore, adipose tissue represents a possible alternative source of multipotent stromal-stem cells (PRUNET-MARCASSUS et al., 2006).

The aims of this study were to analyze and compare the stem cell surface markers expression profiles and the differentiation potential into the three mesodermal lineages of ASVF isolated from two different adipose depots in mice.

Material and Methods

Isolation and culture of ASVFs

Adipose tissue from 8 to 12 week old BALB/c mice were collected from the visceral omentum and gonodal epididymis regions. Tissues were separately minced and digested in collagenase solution (1mg/mL) for 30 min at 37 ° C. After a period of digestion, followed by collagenase inactivation with growth medium (Dulbecco's Modified Eagle Medium-F12 + 10% fetal bovine serum + 100µg (100 IU) of penicillin and, streptomycin + 0,25µg of amphotericin B), cells were centrifuged (200 *xg*, 10 min) to obtain a pellet. Cell pellets were resuspended in growth medium, and the cell suspensions were cultured on cell culture plates in a 5% CO_2 incubator at 37 ° C. The cells were maintained in growth medium and passaged using trypsin solution (0.25%) once they achieved 70- 80% confluency.

Adipogenic, osteogenic, and chondrogenic differentiation

At passage 6 (P6), cultures of ASVFs from the omentum (OASVF) and epididymis (EASVF) were submitted to differentiation protocols using osteogenic medium (50 µM L-ascorbic acid 2-phosphate, 0.1 µM dexametasone, and 15 mM β-glycerolphosphate), chondrogenic medium (50 µM dexametasone, 50 µM L-ascorbic acid 2-phosphate, 10 ng/mL TGF-β, and 1x insulin-transferrin-sodium selenite), and adipogenic medium (50 µM indomethacin, 1 µM rosiglitazone, 1µM dexamethasone, and 1µg/mL insulin) at 17, 21, and 26 days. Each medium was changed every 3 days and histological staining was performed after the differentiation period. Cells induced with osteogenic, adipogenic, and chondrogenic media were first fixed with 4% paraformaldehyde and then stained with alizarin Red (pH 4.1), oil red, and alcian blue (pH 2.5), respectively. After staining, cells were observed using an inverted microscope (Leica DMI600B).

Flow cytometric analysis

OASVF and EASVF cultures at the passages 2 (P2), 4 (P4), and 7 (P7) were analyzed by flow cytometry (FACS) for stem cells surface markers CD45, CD73, and CD105. Briefly, cells were trypsinized and, centrifuged (200 *xg*, 10min), and 4 x 10^4 cells were suspended in stain buffer (BD Biosciences) in microtubes. Samples were then incubated with the antibodies CD45-FITC (1.5 µg/µL), CD 73-FITC (1 µg/µL), and CD105-PE (1 µg/µL) (BD

Biosciences) in the dark for 20 min at 37° C. After incubation, cells were analyzed using a flow cytometer (BD FACSuite).

Animals

All procedures using BALB/c mice in the present study were approved by the Institutional Committee for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil, approval number 087/2014.

Results

Differentiation potential of ASVFs

After the differentiation period, both adipose tissues were able to differentiate into all three linages. However, histological staining showed that osteogenic and chondrogenic differentiation occurred earlier in OASVF; after 21 days of differentiation the cultures stained positive (Figure 1). For EASVF, 26 days of differentiation were needed to obtain consistent positive staining (Figure 1). Cells derived from stromal fractions and maintained in osteogenic medium formed aggregates or nodules, and these bone nodules stained positive with alizarin red (Figure 1). With chondrogenic differentiation, cells developed a multilayered matrix that was strongly stained with alcian blue, indicating an abundance of glycosaminoglycans in the extracellular matrix (Figure 1). EASFV cultures showed several areas that were more strongly stained with alizarin red, and alcian blue than OASVF, indicating that epididymis adipose-derived cells more effectively differentiated into osteogenic and chondrogenic lineages (Figure1). In adipogenic differentiation, both adipose tissue cultures needed 17 days to differentiate and showed an accumulation of lipid-rich vacuoles within cells. These vacuoles were positively stained by oil red staining (Figure 1).

Expression profiles of stem cell surface markers in ASVFs

This study analyzed two sources of ASVF cells, based on the expression of surface antigens: CD45, a hematopoietic linage marker, CD105 and CD73, surface proteins expressed in mesenchymal stem cell (MSC). Flow cytometric analysis (FACS) showed an increase in $CD105^+$ and $CD73^+$ single-positive cells and, a decrease in double-positive $CD73^+/CD105^+$ cells in P2, P4, and P7 OASVF (Figure 2 and Table 1). Conversely, EASVF showed a

tendency toward an increase in the double-positive $CD73^+/CD105^+$ cells, as well as an increase in the MSC $CD105^+$ cells in the same passages mentioned above (Figure 2 and Table 2). In both ASVFs, cells expressed insignificant levels of CD45 (Table 1 and 2), and, in EASVF, the levels of $CD105^+$ significantly increased with higher passages (Table 2).

Discussion

Depending on the fat location, adipose tissue-derived cells have unique characteristics related to metabolic properties, functions, gene expression profiles, and differentiation potential (SCHÄFFLER & BÜCHLER, 2007; TCHKONIA et al., 2002). We analyzed ASFVs from two different fat pads in mice, the omentum and epididymis, and although the mesenchymal surface markers were expressed in cells from both sources, their levels differed between cells from the two adipose sources and among passages. In OASFV, there was a decrease in double-positive $CD105^+/CD73^+$ cells, while in EASVF there was an increase in cells expressing the double-positive mesenchymal surface markers in P2, P4, and P7. Flow cytometry showed that a higher number of passages was needed to achieve significative $CD105^+$ MSC populations in EASVF cultures, probably because selection and expansion of AT-MSCs occurred with each passage. Previous immunophenotyping study of MSCs revealed heterogeneity at early stages within cell populations before an eventual subpopulation was selected through extensive cultivation (DA SILVA et al., 2006). Taken together, the results indicated that adipose tissues isolated from mouse omentum and epididymis have different subsets of MSC, suggesting that they are sources of different somatic stem cells.

ASFV immunophenotype results presented in this study are in accordance with results of other studies that relate the variable expression of surface markers to differences in tissue sources, the method of isolation and culture, and species differences (CHAMBERLAIN et al., 2007, DA SILVA et al., 2006; PRUNET-MARCUSSUS et al., 2006; SCHÄFFLER & BÜCHLER, 2007). Interestingly, previous studies have demonstrated that the expression of surface antigens, CDs, also show significant variation during subculture. For example, studies comparing freshly isolated human adipose-derived cells and serially passaged AT-MSCs showed a progressive increase in mesenchymal markers like CD90, CD73, and CD29 (MOSNA et al., 2010). Similar increases occurred in the present study in the expression of MSC surface markers CD105 and CD73 in EASFV at P2, P4, and P7. However, decreases in surface markers have also been observed. da Silva et al. (2006) reported that there was a

tendency toward a decrease in CD117, a stem cell factor receptor, during serial passage of MSCs. Taken together these results illustrate that stem cell surface antigen expression profiles vary with different cell sources and passages.

Although ASVF from both the omentum and epididymis showed the potential to differentiate into adipogenic, osteogenic, and chondrogenic lineages, OASVF differentiated earlier into chondrogenic and osteogenic linages (at 21 days of culture) than the epididymis which required 26 days. The differentiation shown in the present study suggests that mouse epididymis adipose-derived cells have greater potential to differentiate into osteogenic and chondrogenic lineages than omentum adipose-derived cells. These results are in agreement with a previous study that showed, in a murine system, evidence that the differentiation capacity of the SVF is heterogeneous, and varies according to the localization of the adipose tissue (SCHÄFFLER & BÜCHLER, 2007). In rabbits, the osteogenic potential of adipose-derived stem cells (ADSC) isolated from visceral adipose tissue was reported to show greater potential for differentiation compared with ADSCs isolated from subcutaneous adipose tissue (SCHÄFFLER & BÜCHLER, 2007). Prunet-Marcussus et al. (2006) showed that mouse inguinal adipose-derived cells showed a greater capacity to differentiate into osteogenic lineage than did cells from an epididymis adipose fat source.

Since different fat pads have their own metabolic characteristics, fatty acid compositions, and gene expressions (SCHÄFFLER & BÜCHLER, 2007; TCHKONIA et al., 2002), the source of adipose tissue might be expected to influence characteristics of AT-MSCs, such as surface markers and differentiation potential, as shown here. Therefore, additional studies are necessary to properly understand the cellular composition and molecular characteristics, as well as the plasticity, of SVF cells isolated from different fat depots.

Conclusion

This study demonstrated that omentum and epididymis fat pads represent different sources of adipose-derived mesenchymal stem cells, which exhibit diverse surface marker expression profiles when maintained in culture, as well as, distinct differentiation potentials into osteogenic, chondrogenic lineages. Therefore, different adipose tissue sources provide different stromal multipotent stem cells.

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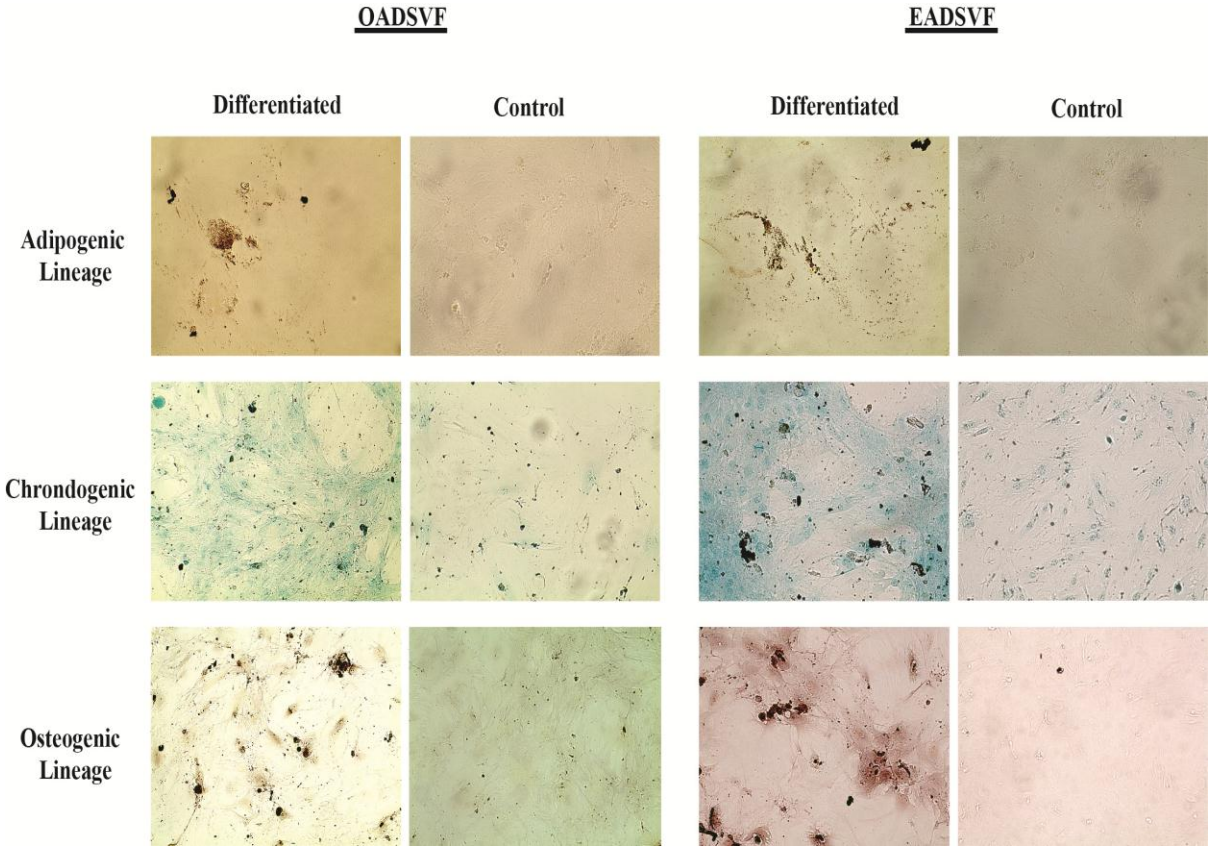

Figure 1 – Differentiation of mouse adipose tissue-derived stromal vascular fraction cells from omentum (OADSVF) and epididymis (EADSVF) in osteogenic, chondrogenic and adipogenic lineages. (Magnification 100 X)

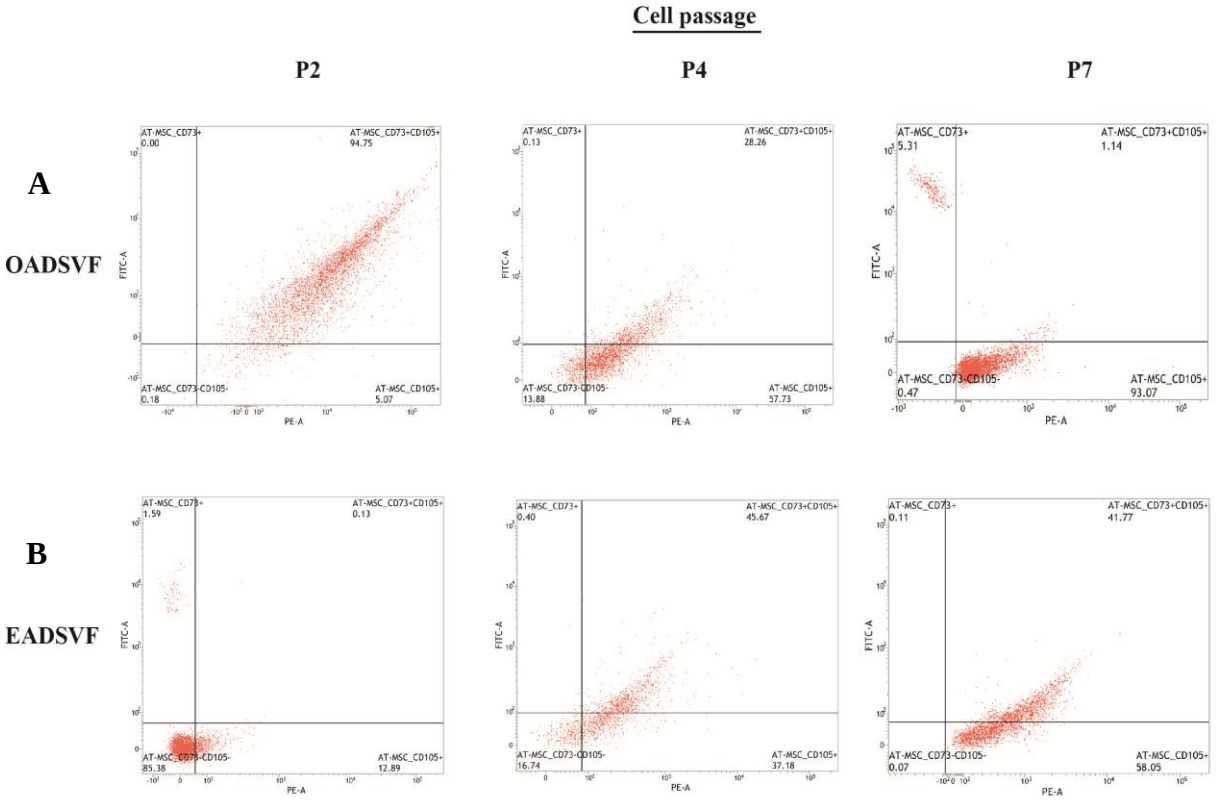

Figure 2 – Flow cytometric analysis of the MSC markers CD73 and CD105 in mouse adipose tissue-derived stromal vascular fraction cells from omentum (OADSVF) (A) and epididymis (EADSVF) (B), at three different passages. P2 (cell passage number 2), P4 (cell passage number 4) and P7 (cell passage number 7)

Table 1 – Flow cytometric analysis of the expression of MSC markers CD73 and CD105, and hematopoietic marker CD45 in mouse OADSVF at various passages

Cell surface marker	OADSVF cell passage 2 (P2)	OADSVF cell passage 4 (P4)	OADSVF cell passage 7 (P7)
CD 45^{+}/CD105^{-}	0.0%	0.28%	0.0%
CD 45^{-}/CD105^{+}	99.31%	77.07%	79.82%
CD45^{+}/CD105^{+}	0.67%	8.38%	1.34%
CD73^{+}/CD105^{-}	0.0%	0.13%	5.31%
CD73^{-}/CD105^{+}	5.07%	57.73%	93.07%
CD73^{+}/CD105^{+}	94.75%	28.26%	1.14%

OADSVF: adipose tissue-derived stromal vascular fraction cells from omentum.

Table 2 – Flow cytometric analysis of the expression of MSC markers CD73 and CD105 and hematopoietic marker, CD45 in mouse EADSVFs at various passages

Cell surface markers	EADSVF cell passage 2 (P2)	EADSVF cell passage 4 (P4)	EADSVF cell passage 7 (P7)
CD 45^{+}/CD105^{-}	0.04%	0.13%	0.0%
CD 45^{-}/CD105^{+}	12.55%	76.73%	92.43%
CD45^{+}/CD105^{+}	0.18%	8.43%	4.63%
CD73^{+}/CD105^{-}	1.59%	0.40%	0.11%
CD73^{-}/CD105^{+}	12.89%	37.18%	58.05%
CD73^{+}/CD105^{+}	0.13%	45.67%	41.77%

EADSVF: adipose tissue-derived stromal vascular fraction from epididymis

4 CAPÍTULO II – CHARACTERIZATION OF ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS AND THEIR DIFFERENTIATION POTENTIAL INTO MALE GERM CELLS AFTER TREATMENTS WITH RETINOIC ACID AND CONDITIONED MEDIUM

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Abstract

Mesenchymal stem cells are more plastic than expected when cultured under certain conditions and the adipose tissue is a reliable source of mesenchymal stem cells that can transdifferentiate into multilineage cells. In the present study, adipose tissue-derived mesenchymal stem cells (AT-MSC) were isolated from omentum and epididymis fat depots, and, previously characterized based on stem cell surface markers and on the mesodermal trilineage differentiation potential. Additionally, both AT-MSCs were cultured with differentiation media containing retinoic acid (RA) and/or testicular cell-conditioned medium (TCC). The AT-MSCs expressed mesenchymal surface markers CD73 and CD105 and differentiated into adipogenic, chondrogenic and osteogenic lineages. After the differentiation treatments, omentum-derived AT-MSCs expressed a gene marker related to male germ cell lineages. These findings reaffirm the importance of adipose tissue as a source of multipotent stromal-stem cells for differentiation researches.

Keywords: *Gdnf* expression. *In vitro* differentiation. Mesenchymal stem cells. Retinol-derivative. Testicular cell-conditioned medium.

Introduction

Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are multipotent cells which proliferate, *in vitro*, for indefinite period and can be induced to differentiate into all three germ layers, which develop into bone, cartilage, fat, muscle, heart, neural, and other cells (ZUK et al., 2002). In addition, adipose tissue is abundant and accessible, representing a reliable source of stem cells which exist in significant numbers in this tissue (FRASER et al., 2007; GIMBLE et al., 2007; MITCHELL et al., 2006).

An area of biological research that generates great optimism is the use of stem cells for the treatment of diseases. Much of the excitement centers on embryonic stem cells (ES), however, this approach remains controversial for ethical reasons and also due the risk of teratomas. Moreover, realization of this strategy as routine clinical application requires extensive research (NAYERNIA et al., 2006; YOUNG et al., 2004). In contrast, AT-MSCs from adult adipose tissues are well characterized, easy to isolate, and have recently been used for therapeutic applications (FRASER et al., 2007; GIMBLE et al., 2007; MITCHELL et al., 2006).

In vitro differentiation of AT-MSCs has demonstrated promising plasticity (ZUK et al., 2002). Recent studies have revealed several substances that can act as differentiation inducers, and generally, they are used in association with one another (MOSNA et al., 2010; SCHÄFFLER & BÜCHLER, 2007). Retinoic acid (RA) is a vitamin A (retinol)–derivative that is widely used as a differentiation inducer. In some studies, RA has been added to culture medium for differentiation of mesenchymal stem cells (MSCs) (HUANG et al., 2010; NAYERNIA et al., 2006) and skin-derived stem cells (TAN et al., 2016) into germ cells. Furthermore, the conditioned medium obtained from the cell culture supernatants is applied in association with chemical inducers to improve differentiation. Conditioned medium has been employed for chondrogenic, myogenic, neural, and germ cell differentiation of bone marrow- and adipose tissue-derived MSCs (DA SILVA et al., 2013; HAN et al., 2014; STERN-STRAETER et al., 2013).

Several methods have been used to investigate *in vitro* and *in vivo* MSCs differentiation. Analyses of marker gene transcripts allow confirmation of AT-MSC differentiation and facilitate the selection of efficient inducers (HOU et al., 2014; PHINNEY & PROCKOP, 2007; SCHÄFFLER & BÜCHLER, 2007). The genes commonly investigated to confirm MSCs differentiation into male germ cell are *Vasa*, *Stella*, *Dazl, Stra8*, *Nanos2*, *Plzf*, and *Gdnf* which is used as a marker for Sertoli cells (CHEN & LIU, 2015; HOU et al., 2014 IKAMI et al., 2015).

Taking these data into account, the main aim of the present study was to evaluate the expression of relevant gene markers of germinative cells in omentum and epididymis -derived AT-MSCs, after treatments with RA and testicular cell-conditioned medium.

Material and Methods

Isolation and culture of AT-MSCs

Adipose tissue from 8 to 12 week old BALB/c mice, was collected from the omentum and epididymis regions. The tissue was, separately, minced and digested in collagenase solution (1 mg/mL) for 30 min at 37 ° C. After digestion, the collagenase solution was inactivated with growth medium (Dulbecco's Modified Eagle Medium-F12 + 10% fetal bovine serum + 100μg (100 IU) of penicillin and, streptomycin + 0,25μg of amphotericin B) and the cells were centrifuged (200 *xg*, 10min) to obtain a pellet. The resulting pellet was resuspended in growth medium, and the cell suspension was cultured in cell culture plates in a 5% CO_2 incubator at 37 ° C. The cells were maintained in growth medium and passaged using trypsin solution (0.25%) once they achieved 70-80% confluency.

Testicular cell-conditioned medium preparation

Testicles from 8 to 12 week old BALB/c mice were removed, minced, and enzymatically digested in collagenase solution (1 mg/mL) for 30 minutes at 37° C. Tissue digestion was terminated with addition of growth medium for collagenase inactivation, and then the tissue homogenate was centrifuged for 10 min at 200 *xg* and the resulting pellet was resuspended in growth medium. The testicular cells were cultured in 5% CO_2 at 37 ° C and passaged using trypsin solution (0.25%) once they reached 80-90% confluency. Seven days after cultures were started, and every 3 days afterward for 30 days, testicular cell-conditioned medium (TCC) was collected. After collection, the TCC was centrifuged (200 *xg*, 10 min), and the supernatant was filtered (0.22 μm) and stored at - 20°C until use.

Differentiation treatments of AT-MSCs

Cultures of AT-MSCs derived from the omentum and epididymis fat pads were induced by differentiation medium containing growth medium supplemented with 10^{-6}M RA (Sigma), 50% TCC + 50% growth medium, or RA + TCC at 7, 14, and 21 days. Adipose tissue-derived cells maintained with only growth medium were used as a control group. The media were changed every 3 days.

Adipogenic, osteogenic and chondrogenic induction of AT-MSCs

Omentum and epididymis-derived AT-MSCs were cultured in osteogenic medium (50 µM L-ascorbic acid 2-phosphate, 0.1 µM dexametasone, and 15 mM β-glycerolphosphate) and chondrogenic medium (50 µM dexametasone, 50 µM L-ascorbic acid 2-phosphate, 10 ng/mL TGF-β, and 1x insulin-transferrin-sodium selenite) for 21 days, and with adipogenic medium (50 µM indomethacin, 1 µM rosiglitazone, 1µM dexamethasone, and 1µg/mL insulin) for 17 days. The media were changed every 3 days and, histological staining was performed after the differentiation period. Cells induced with osteogenic, adipogenic, and chondrogenic media were first fixed with paraformaldehyde 4% and then stained with alizarin red (pH 4.1), oil red, and alcian blue (pH 2.5), respectively, and observed using an inverted microscope (Leica DMI600B).

Flow cytometry analysis

AT-MSCs from omentum and epididymis fat depots were trypsinized, centrifuged, and then 4 x 10^4cells were suspended in Stain Buffer (BD Biosciences) in separate microtubes. Samples were then incubated with the antibodies CD45-FITC (1.5µg/µL), CD73-FITC (1µg/µL), and CD105-PE (1µg/µL) (BD Biosciences) in the dark for 20 min at 37° C. After incubation, cells were analyzed by flow cytometry using the BD FACSuite (BD Biosciences).

RNA isolation, reverse transcription, and real-time PCR

After each differentiation induction period (7, 14, and 21 days), total RNA was isolated from the cell cultures. RNA extraction was performed using Trizol reagent (Invitrogen) according to the manufacturer' s instructions. After extraction, RNA concentration and quality were checked by a NanoDrop1000 spectrophotometer (Absorbance of 260/280 nm) (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 1000 ng of RNA, which was first treated with 0.1 U of DNase Amplification Grade (Life Techonologies) for 5 min at 37° C. After DNase inactivation at 65 °C for 10 min, samples were incubated in 20µLwith reagents from an iScript cDNA synthesis Kit (BioRad). cDNA synthesis was performed in three steps: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

The relative expression levels of specific genes were determined by quantitative PCR (qPCR) conducted in a CFX384 thermocycler (BioRad) using GoTaq qPCR Master Mix

(Promega). Expression of the murine *Vasa*, *Stella*, *Dazl*, *Stra8, Nanos2*, *Plzf* and *Gdnf* genes were analyzed, in addition the murine housekeeping genes *Gapdh* and *β-Actin*. All primers were designed based on gene sequences deposited in the GenBank database using Primer Express Software (Applied Biosystems). Table 1 shows the genes and primer sequences used for the qPCR analysis.

Standard two-step qPCR was performed in a 10 µL final volume containing 2 µL of cDNA, 2x Master Mix (Promega) and 5 µM each primer. Amplification conditions were 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 60 °C for 1 min. Melting-curve analyses were performed to verify product identity. To optimize the qPCR assay, serial dilutions of the cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95% and 105% were considered optimized. The relative standard curve method was used to quantify transcripts in each sample (CIKOS et al., 2007). Samples were run in duplicate, and results were expressed relative to the average Ct values for the *Gapdh, β-Actin* genes as internal controls. Samples of mRNA extracted from mouse testicles were used as a positive control for validating primers and amplicons.

Animals

All procedures using BALB/c mice in the present study were approved by the Institutional Committee for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil, approval number 087/2014.

Results

AT-MSCs characterization

Flow cytometric analysis demonstrated that omentum-derived AT-MSCs predominantly expressed the surface marker CD105 and showed low levels for CD73 and double-positive CD105/CD73 cells (Figure 1); CD105 and CD103 are typically found on MSCs surface. Epididymis-derived AT-MSCs showed prevalent population of double-positive CD73/CD105 cells, as well as CD105 cells .Omentum and epididimis-derived AT-MSCs lacked CD45 surface expression, which is seen in the hematopoietic lineage (Figure 1).

Differentiation protocols were used to induce AT-MSCs from both mouse fat depots in bone, cartilage, and fat to further confirm their trilineage differentiation capacity. After culture of AT-MSCs in osteogenic medium, the cells differentiated into osteoblasts, with calcium accumulation indicated by positive staining with alzarin red (Figure 2). AT-MSCs maintained in adipogenic medium showed the presence of intracellular lipid droplets, confirmed by oil red staining (Figure 2). Chondrogenic differentiation was indicated by alcian blue staining of abundant glycosaminoglycans within the extracellular matrix (Figure 2). The AT-MSCs grown in culture medium (undifferentiated) did not show any lipid droplets and maintained their typical fibroblast-like shape (Figure 2).

Evaluation of gene expression in treated AT-MSCs

A qPCR assay was performed to determine the gene expression levels. Among the genes and sources of AT-MSCs tested, only *Gdnf* was expressed just in omentum-derived AT-MSCs treated with RA differentiation media after incubation periods of, 7, 14, and 21 days (Figure 3). Conversely, no significant expression was detected in AT-MSCs maintained in TCC medium for any period of treatment (Figure 3). *Gdnf* gene expression was not detected in untreated AT-MSCs (Figure 3).

Discussion

AT-MSCs are stromal stem cells that can differentiate into all three germ layers under suitable conditions and recent studies have shown that somatic adult stem cells are more plastic than previous expected (PHINNEY & PROCKOP, 2007; ZUK et al., 2002). Therefore, in the present study, the surface antigen expression of adipose tissue-derived cells indicated that MSCs represent a significant population among the cells isolated from both, omentum and epididymis, fat sources. Additionally, adipose tissue-derived cells from both the omentum and epididymis showed the potential to differentiate into the three mesodermal, adipogenic, osteogenic, and, chondrogenic, lineages.

The omentum-derived AT-MSCs expressed high levels of CD73 and CD105, which are MSCs markers, and were negative for CD45 surface antigen, a hematopoietic lineage marker. In addition, the effective differentiation of omentum-derived AT-MSCs into all three lineages (adipogenic, osteogenic, and chondrogenic) confirmed the presence of AT-MSCs and their differentiation potential.

The qPCR results suggest that RA and RA+TCC stimulated *Gdnf* expression in omentum-derived AT-MSCs. Conversely, AT-MSCs maintained in TCC did not express the target gene, similar to untreated cells. Taken together, these results indicate that RA was the main factor involved in *Gdnf* expression.

RA has been used in association with other substances to induce neuronal differentiation from cultured mouse AT-MSCs (BI et al., 2010; PAVLOVA et al., 2012). However, RA alone has usually been used to promote the differentiation of embryonic stem cells (ESCs) and MSCs into germ cells (DRUSENHEIMER et al., 2007; GEIJSEN et al., 2004; KERKIS et al., 2007; NAYERNIA et al., 2006). An active derivative of vitamin A, RA influences germ cell differentiation and is required for the transition to meiosis in both female and male germ cells (KOUBOVA et al., 2006). Retinoids are involved in the regulation of testicular functions, which appear to be necessary for spermatogenesis (LIVERA et al., 2002). RA receptors (RARs) are expressed in both Sertoli and germ cells (ESKLID et al., 1991), and RA functions inside the nucleus, recognizing two different classes of RARs. Both classes (RARs and RXRs) consist of three types of receptors, α, β, and γ, encoded by distinct genes, and they transduce RA signals by binding directly to RA-responsive elements (ROSSI & DOLCI, 2013). Previous studies have indicated that RA favors spermatogonial differentiation through direct action on spermatogonia and indirect action mediated by changes in the expression of GDNF secreted by Sertoli cells (ROSSI & DOLCI, 2013).

The *Gdnf* gene is mainly expressed in Sertoli and neuronal cells and is considered a glial marker that characterizes neuronal differentiation of stem cells (BI et al., 2010; SHAKHBAZOV et al., 2009). Additionally, GDNF is a paracrine soluble factor secreted by Sertoli cells in the testicular niche that influences the self-renewal of spermatogonial stem cells (SSCs) and inhibit their differentiation (CHEN & LIU, 2015; IKAMI et al., 2015; ROSSI & DOLCI, 2013). Therefore, based on the effect of RA on *Gdnf* gene expression showed in this study, it seems that omentum AT-MSCs contain RA receptors, and that they respond to RA stimulation, but further studies are necessary to properly characterize the RARs in AT-MSCs.

The testes are abundant sources of numerous hormones and growth factors, such as bone morphogenic protein 4 (BMP4), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), stem cell factor (SCF), growth differentiation factor-9 (GDF9), and testosterone, all of which are needed for the development of male germ cells (CREEMERS et al., 2002; PELLEGRINI et al., 2003; TAKABAYASHI et al., 2001). Recent studies have shown that TCC supports the differentiation of ESCs into germ cells (LACHAM-KAPLAN

et al., 2006). However, in the present study, TCC was not effective promoting gene expression maybe, due the inappropriate concentration of the soluble factors. Therefore, it seems that TCC might be used in combination with other induction factors and might be concentrated to improve the efficiency of differentiation.

MSCs are considered stromal adult stem cells and their potential to differentiate into stromal lineages has been demonstrated. However, there are not studies considering the AT-MSCs differentiation into Sertoli cells which are stromal cells that support spermatogenesis in adult males (SCHÄFFLER & BÜCHLER, 2007; ZUK et al., 2002). Although previous studies have shown that MSCs maintained in media containing RA differentiate into male germ cells (DRUSENHEIMER et al., 2007; NAYERNIA et al., 2006; ZHANG et al., 2014) and that TCC enhances this differentiation (HUANG et al., 2010; KAVIANI et al., 2014) additional gene expression and other analyses should be performed to confirm whether the AT-MSCs treated with RA, in the present study, can differentiate into cell lineages related to male germ cells.

Conclusion

This study demonstrated that mouse adipose tissue-derived cells contain a significant AT-MSC population. In addition, omental AT-MSCs express *Gdnf,* an important marker of male germ cells, after treatment with RA. This observation indicates that omental-derived AT-MSCs respond to differentiation treatments therefore, they serve as a suitable source of multipotent stem cells for further differentiation studies.

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Table 1 – List of primer sequences and accession numbers for gene sequences used for the mRNA gene expression analysis

Gene	Forward primer	Reverse primer	Accession number
Dazl	CGAAGCATACAGACAGTGGTCTCT	TAAGCACTGCCCGACTTCTTCT	010021.5
Stra8	TTGCCGGACCTCATGGAAT	GTGTCACTTCATGTGCAGAGATGAT	009292.1
Stella	CGGTGCTGAAAGACCCTATAGC	GGCTCACTGTCCCGTTCAAA	139218.1
Vasa	GGCTGTGTTTGCATCTGTTGAC	ATCAACTGGATTGGGAGCTTGT	001145885.1
Nanos2	AGGTAGCTGAGGAGCCCAACTC	TGCTTGCAGAAGTTGCATATGG	194064.2
Plzf	CGAGCTTCCGGACAACGA	AAATGCATTCTCAGTCGCAAAC	001033324.2
Gdnf	GATTCGGGCCACTTGGAGTT	GACAGCCACGACATCCCATAA	010275.2
Gapdh	CAGCCTCGTCCCGTAGACAA	GTAGACCATGTAGTTGAGGTCAATGAA	008084.2
β-Actin	TCGTGGGCCGCTCTAGGCAC	TGGCCTTAGGGTTCAGGGGG	007393.3

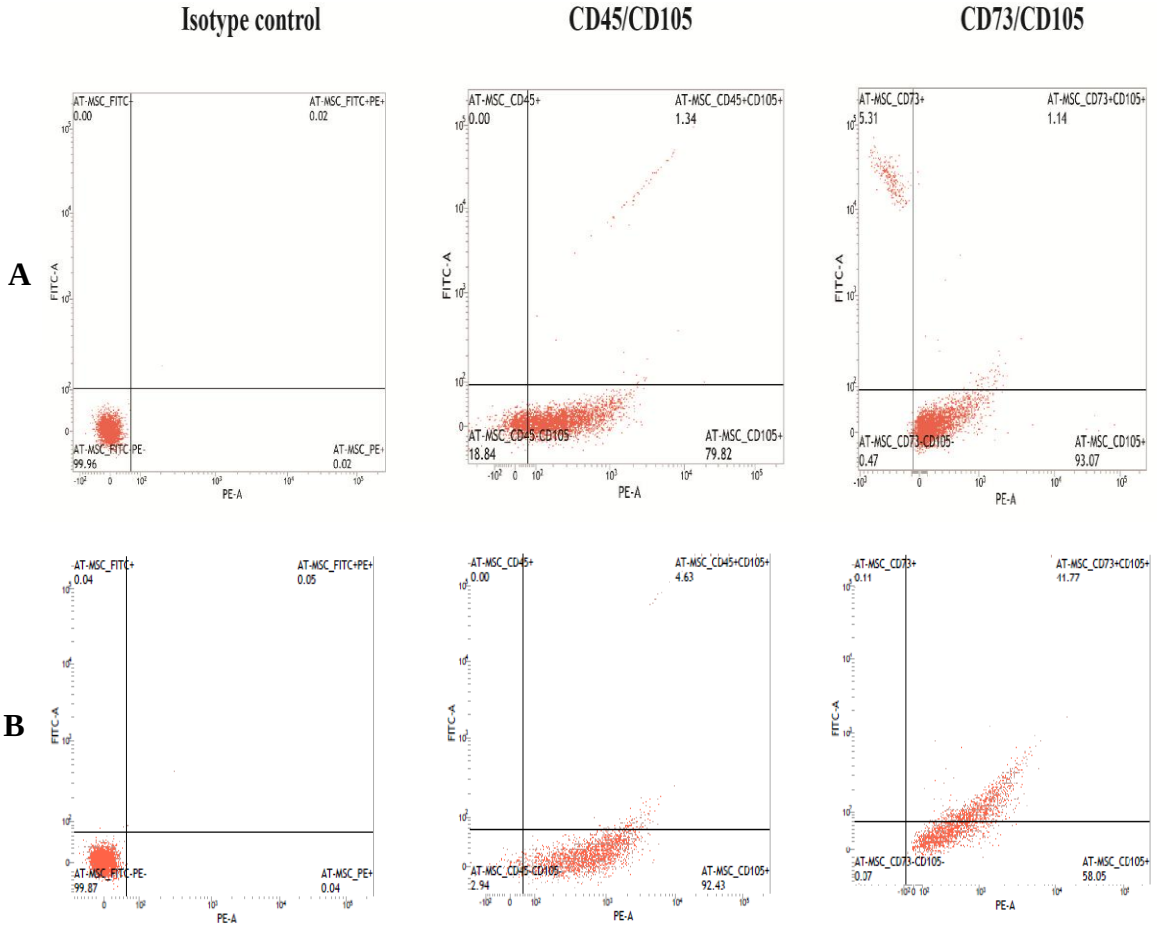

Figure 1 – Flow cytometric analysis of cell surface markers CD73, CD105 and CD45 in adipose tissue-derived mesenchymal stem cells from mouse omentum (A) and epididymis (B)

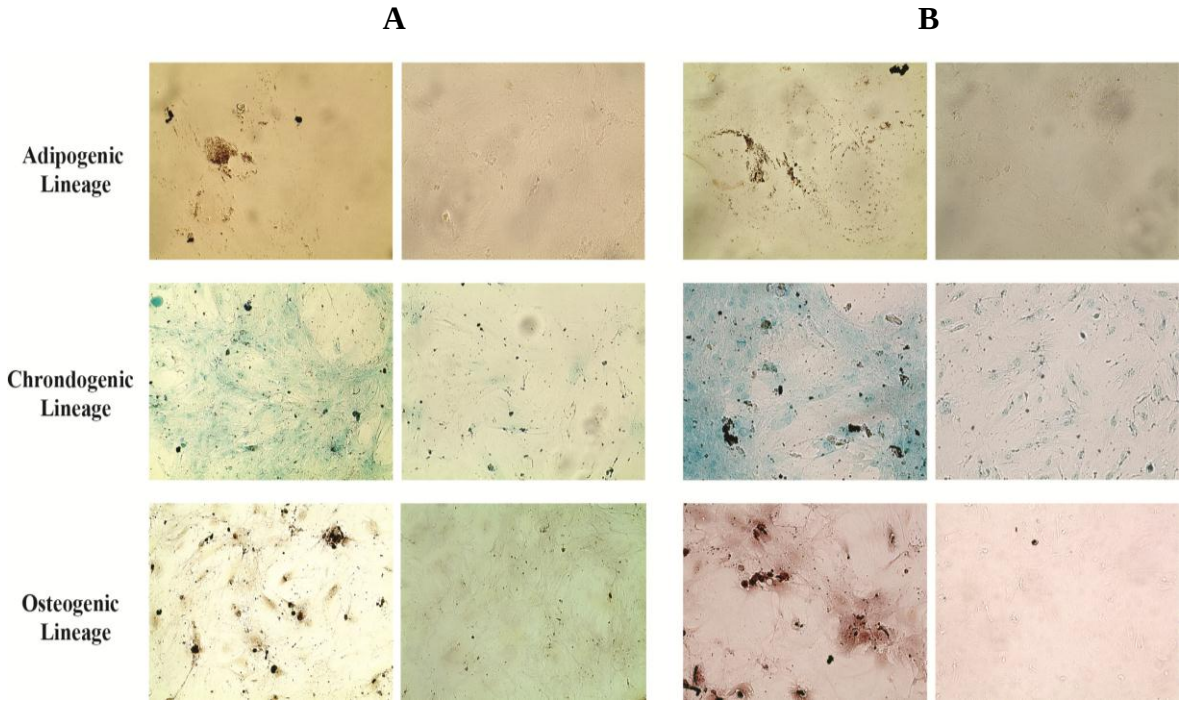

Figure 2 – Mesodermal differentiation of adipose tissue-derived mesenchymal stem cells from mouse omentum (A) and epididymis (B). (100X magnification)

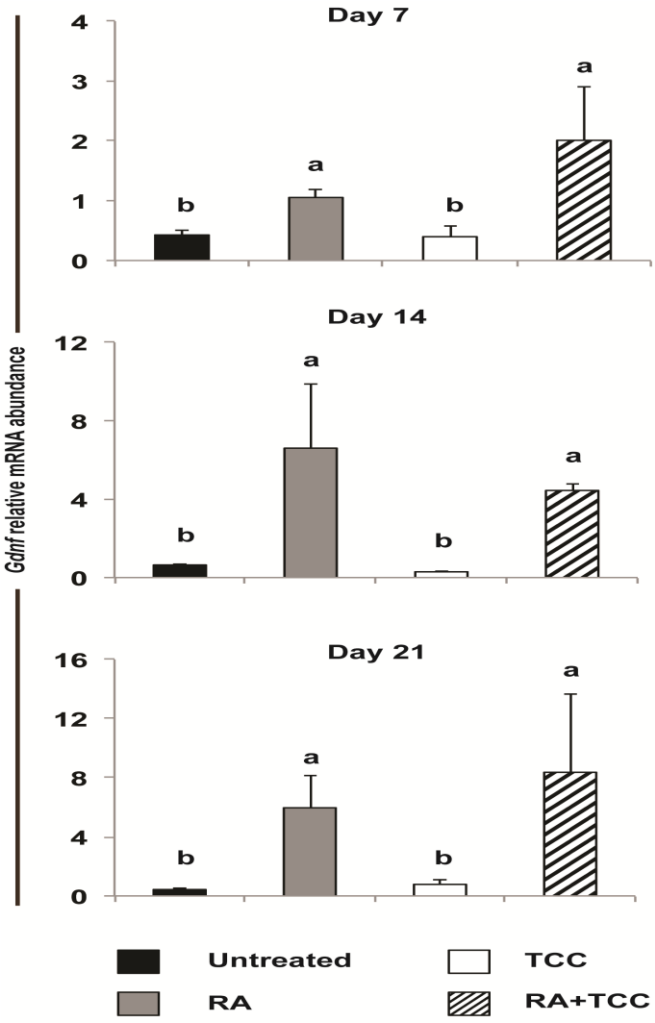

Figure 3 – Relative expression of the murine *Gdnf* gene in omentum AT-MSCs treated with retinoic acid (RA), testicular cell-conditioned medium (TCC), or RA+TCC for 7, 14, and 21 days, or with growth medium only for an untreated control group. Values with different superscripts (a, b) are significantly different ($p < 0.05$)

5 DISCUSSÃO

Com base nas promissoras características das células-tronco mesenquimais derivadas do tecido adiposo (AT-MSCs) (SCHÄFFLER & BÜCHLER, 2007; ZUK et al., 2002) o presente estudo pesquisou a presença, as características e o potencial de diferenciação das AT-MSCs isoladas do tecido adiposo depositado em diferentes regiões do organismo de camundongos BALB/c. Uma vez que o tecido adiposo encontra-se amplamente distribuído no organismo dos mamíferos, estando localizado preferencialmente na região subcutânea e em contato com os órgãos e gônadas (BUNNELL et al., 2008; STREM et al., 2005), nesse estudo elegeu-se coletar o tecido adiposo da região do omento e testículos devido à facilidade de obtenção, assim como devido à quantidade significativa de gordura depositada nessas regiões.

No primeiro estudo, foram isoladas e caracterizadas as AT-MSCs provenientes dos dois diferentes depósitos de gordura acima citados e ambas as fontes demonstraram possuir populações celulares que expressaram os marcadores de superfície mesenquimais CD73 e CD105 e foram negativas para o marcador de linhagens hematopoiéticas CD45. Da mesma forma, as células derivadas da fração estromal vascular de ambas as fontes, quando mantidas em cultivo, demonstraram potencial de diferenciação condrogênica, osteogênica e adipogênica. Esses resultados demonstraram que o tecido adiposo próximo às regiões do omento e dos testículos possui células-tronco mesenquimais na sua composição. Porém, as AT-MSCs isoladas das distintas fontes apresentaram diferentes padrões e níveis de expressão dos marcadores de superfície mesenquimais em distintas passagens *in vitro*. Bem como, o potencial de diferenciação condrogêncio e osteogêncio divergiu levemente entre as fontes, demonstrando que apesar de ambos os tecidos adiposos possuírem AT-MSCs essas revelaram diferentes características conforme sua origem. Os resultados obtidos neste estudo estão de acordo com estudos prévios que também demonstraram variabilidade nos perfis de expressão dos marcadores de superfície nas AT-MSCs conforme o número da passagem celular e a fonte de tecido adiposo, assim como relataram diferença no potencial de diferenciação osteogênica das AT-MSCs dependendo da origem do tecido adiposo (CHAMBERLAIN et al., 2007; MOSNA et al., 2010; PRUNET-MARCUSSUS et al., 2006; SCHÄFFLER & BÜCHLER, 2007).

Uma vez cientes da presença e de algumas características das AT-MSCs reveladas no primeiro estudo, verificou-se o possível potencial de diferenciação das AT-MSCs isoladas do tecido adiposo da região do omento e testículos em linhagens celulares relacionadas às células germinativas masculinas. Para isso, após os períodos de cultivo (7, 14 e 21 dias) com ácido

retinóico e/ou meio condicionado proveniente do sobrenadante do cultivo das células de testículo, pesquisou-se a expressão de genes marcadores específicos relacionados às células germinativas precoces, *Vasa*, *Stella*, *Dazl*, *Stra8, Nanos2, Plzf,* e à célula de Sertoli, *Gdnf* (CHEN & LIU, 2015; HOU et al., 2014; IKAMI et al., 2015). As análises por meio da qPCR revelaram que somente as AT-MSCs isoladas do tecido adiposo em contato com o omento expressaram gene marcador específico, sendo detectado unicamente a expressão do gene *Gdnf*. Contudo, mais análises necessitam ser realizadas para confirmar uma possível diferenciação das AT-MSCs em linhagens celulares relacionas às células reprodutivas masculinas.

Além disso, os resultados obtidos demonstraram que o ácido retinóico é o fator de indução da expressão do gene *Gdnf* e o meio condicionado testicular, somente quando combinado ao ácido retinóico, e em determinados períodos do tratamento (7 e 21 dias), promoveu a expressão desse gene, porém o meio condicionado sozinho mostrou-se ineficiente para induzir a expressão do *Gdnf*. Portanto, a composição ou a concentração dos fatores solúveis que constituíram o meio condicionado testicular revelou-se ineficaz na indução da expressão dos genes marcadores específicos relacionados às células germinativas. Resultados semelhantes estão descritos em estudos prévios demonstrando que células-tronco mesenquimais, após serem cultivadas com meios de diferenciação contendo ácido retinóico ou ácido retinóico associado ao meio condicionado testicular, passaram a expressar os genes *Vasa*, *Dazl*, *Stella* e *Stra8,* mas não relataram expressão do *Gdnf* (DRUSENHEIMER et al., 2006; HUANG et al., 2010; ZHANG et al., 2014).

Considerando os resultados obtidos nos estudos que compõem a presente tese, o tecido adiposo possui na sua composição células-tronco mesenquimais com características únicas dependendo da origem desse tecido e que essas AT-MSCs apresentam potencial de diferenciar quando mantidas em meios apropriados contendo fatores de indução de diferenciação. Estes resultados reafirmam o tecido adiposo como fonte de células-tronco multipotentes e estão de acordo com estudos prévios que demonstraram características moleculares, biológicas e a plasticidade das AT-MSC (MOSNA et al., 2010; NAYERNIA et al., 2006; SCHÄFFLER & BÜCHLER, 2007; ZUK et al., 2002).

Portanto, devido ao seu amplo potencial plástico acredita-se que as AT-MSCs possuam inclusive a capacidade de diferenciar em células germinativas masculinas, consequentemente validando sua aplicação em estudos futuros de diferenciação celular para que possam ser utilizadas na terapia celular da infertilidade e outras desordens reprodutivas masculinas, assim como em diversos campos da terapia celular.

6 CONCLUSÃO

A partir dos resultados obtidos nos estudos que compõem a presente tese, pode-se concluir que o tecido adiposo localizado em contato com o omento e o tecido adiposo depositado próximo aos testículos são fontes de distintas células-tronco mesenquimais, uma vez que, as AT-MSCs isoladas dessas regiões demonstraram-se diferentes quanto ao padrão de expressão de marcadores mesenquimais de superfície e quanto ao potencial de diferenciação osteogênico e condrogênico.

Além disto, os resultados demonstram que somente as AT-MSCs isoladas do tecido adiposo da região do omento responderam ao tratamento de diferenciação com ácido retinóico expressando o gene *Gdnf*. Portanto, com base nesse resultado de diferenciação, sugere-se o tecido adiposo da região do omento como o mais indicado a ser empregado em futuras pesquisas para confirmar a diferenciação das AT-MSCs e para identificar a linhagem celular na qual as AT-MSCs possivelmente diferenciaram.

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APÊNDICES

APÊNDICE A – ARTIGO CIENTÍFICO PRODUZIDO DURANTE O PERÍODO DO DOUTORADO EM COLABORAÇÃO COM A PROF[A] FERNANDA SILVEIRA FLORES VOGEL NO LABORATÓRIO DE DOENÇAS PARASITÁRIAS DA UFSM

DNA extraction methods and multiple sampling to improve molecular diagnosis of *Sarcocystis* spp. in cattle hearts

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ABSTRACT

Molecular detection of *Sarcocystis* spp. in tissue samples can be useful for experimental and diagnostic purposes. However, the parasite spreads unevenly through tissues, forming tissue cysts, and the cystic wall is an obstacle in DNA extraction protocols. Therefore, adequate sampling and effective disruption of the cysts are essential to improve the accuracy of DNA detection by PCR. The aims of this study were to evaluate the efficacy of four protocols for DNA extraction from cysts of *Sarcocystis* spp. present in bovine myocardium samples or after their harvest in phosphate saline buffer solution (PBS), as well as determine the effects of single or multiple sampling on the accuracy of molecular diagnosis of sarcocystosis in cattle hearts. Cysts and myocardium samples from nine bovine hearts were randomly distributed to four DNA extraction protocols: kit, kit with modification, DNAzol, and cetyl-trimethyl ammonium bromide (CTAB). Samples were submitted to DNA extraction and PCR as replicates of each heart (simplicate, duplicate and triplicate), and the probability of a true positive diagnostic was calculated. Among the protocols tested, kit with modification was determined to be most suitable for DNA extraction from cysts in PBS (92.6% of DNA detection by PCR); DNAzol resulted in higher DNA detection frequency from bovine myocardium samples (48.1%). Multiple sampling improved molecular diagnosis of *Sarcocystis* spp. infection in cattle hearts, increasing at 22.2% the rate of true positive diagnostic.

Keywords: genomic DNA, isolation protocols, PCR, 18S rDNA, *Sarcocystis* cysts.

INTRODUCTION

Sarcocystis spp. protozoa are among the most prevalent genera of parasites of livestock (Dubey et al. 1989; Dubey and Lindsay 2006). Cattle are currently known to harbor five of these species (*Sarcocystis cruzi*, *Sarcocystis hirsuta*, *Sarcocystis hominis*, *Sarcocystis rommeli* and *Sarcocystis heydorni*) (Dubey et al. 2015, 2016; Gjerde 2016). The prevalence of

bovine sarcocystosis is close to 100% in almost all the regions studied worldwide (Vangeel et al. 2013). Cattle usually act as intermediate hosts (Dubey et al. 2015, 2016; Moré et al. 2013), presenting a chronic infection course and harboring the parasite cystic form in target tissues such as heart, tongue, esophagus, and diaphragm muscle (Dubey et al. 1989, 2015). Humans can be infected by the ingestion of undercooked beef containing *S. hominis*, acting as definitive hosts with intestinal sarcocystosis (Fayer et al. 2015). Recently, Dubey et al. (2015) described the *S. heydorni* life-cycle, including human as a definitive host and experimentally infected calves as intermediate hosts. Thus, diagnosis of *Sarcocystis* species present in cattle meat is a major issue on human health security.

Conventional methods for *Sarcocystis* identification are based on the cyst wall structure observed under light and electron microscopy (Dubey et al. 1989; Gjerde 2016). However, these are time-consuming methods with limited applications for large quantities of samples (Moré et al. 2011). Besides, microscopy may not be reliable for differentiation among *Sarcocystis* species with little morphological variation (Yang et al. 2002). Moreover, nucleic acid based approaches have been recently developed for detection and genotyping of *Sarcocystis* species. Therefore, molecular techniques such as PCR, RAPD-PCR, and PCR-RFLP can be used to identify and differentiate between cysts present in the host tissues (Gjerde 2016; Güçlü et al. 2004; Xiang et al. 2009; Yang et al. 2002). The small subunit ribosomal DNA gene (18S rDNA) demonstrates considerable variability among *Sarcocystis* species. Thus, 18S rDNA has been shown to be an important molecular target and a significant marker for *Sarcocystis* spp. diagnosis and species differentiation (Fischer and Odening 1996; Moré et al. 2011, 2014). Additionally, partial mitochondrial cytochrome c oxidase subunit I gene (CoxI) sequences have been used to distinguish *Sarcocystis* species that infect cattle (Gjerde 2016).

Molecular assays for protozoa infection diagnosis can be greatly improved through more effective techniques of DNA extraction and PCR techniques with higher sensitivity for gene identification (Babaei et al. 2011; Moré et al. 2013; Nantavisai et al. 2007). Previous studies have revealed that both DNA extraction and amplification methods can influence the PCR results (Güçlü et al. 2004; Yang et al. 2002). Despite the high infection rates by *Sarcocystis* spp. found in cattle (Vangeel et al. 2013), parasite DNA cannot be detected by PCR in many samples collected from infected animals, even when tissues considered as privileged sites for the protozoan are used for molecular research (Fukuyo et al. 2002; Moré et al. 2011, 2013), Firstly, parasite cysts may not be present in most tissue sections sampled. Additionally, the protozoa cyst wall structure, whose thickness varies from μm to mm scales among *Sarcocystis* species, comprises a barrier that hinder disruption of the cysts, thereby greatly reducing the yield of DNA extracted from them (Babaei et al. 2011; Dubey and Lindsay 2006; Guy et al. 2003; Nantavisai et al. 2007; Tenter 1995).

The choice of DNA extraction technique is thus a crucial step for molecular assays, and several extraction approaches may be used including traditional laboratorial protocols and commercially available kits. However, each method has its own set of recommendations, limitations, and disadvantages. Therefore, the objectives of this study were to evaluate (a) the efficacy of four DNA extraction protocols for cysts of *Sarcocystis* spp. present in bovine

myocardium samples or after their harvest in phosphate saline buffer solution (PBS), and (b) the effects of single or multiple sampling on the accuracy of molecular diagnosis of sarcocystosis in cattle hearts.

MATERIAL AND METHODS

Tissue and cyst samples

Nine bovine hearts obtained during slaughter at an abattoir from Santa Maria County, Rio Grande do Sul state, Brazil, were used for sampling. Whole hearts were collected and three pieces of 200 mg were taken from each of the nine hearts. Each of these samples was divided in four pieces of 50 mg, which were individually stored at -20 °C until DNA extraction. Additionally, three pieces of 50 g were taken from each heart for fresh examination and cysts collection. The sarcocysts found were harvested in PBS (pH 7.3) for further use.

Fresh examination

Three pieces of 50 g collected from each heart were separately minced in a meat grinder with 20 ml of PBS (pH 7.3). Each sample was filtered using a strainer with gauze into a Petri dish and examined via a stereomicroscope (NIB-100, China) at 100X magnification. Cysts of *Sarcocystis* spp. were identified and isolated. After identification, 40 cysts from each piece of myocardium were harvested with a micropipette and distributed in aliquots of ten cysts per microtube in solution with 200 µL of PBS. These aliquots were stored at -20 °C until DNA extraction.

DNA extraction protocols

Four aliquots of 10 cysts suspended in PBS, obtained from each myocardium sample, were randomly destined to DNA extraction by the four different protocols tested (Table 1). Additionally, four pieces of 50 mg, cut from each myocardium sample, were randomly destined to each DNA extraction protocol (Table 2).

Protocol 1 (kit)

Genomic DNA was extracted using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) according to manufacturer´s instructions. Briefly, 600 µL of chilled Nuclei Lysis Solution was added to the pools of 10 cysts or 50 mg of myocardium samples, homogenized, and incubated at 65 °C for 30 min. After incubation, 3 µL of RNase Solution was added, incubated for 30 min at 37 °C, followed by addition of 200 µL of Protein Precipitation Solution, vortexed, chilled on ice for 5 min, and centrifuged at 13000 × *g* for 4 min. The supernatant was transferred to a fresh tube containing 600 µL of isopropanol, mixed and centrifuged at 13000 × *g* for 1 min. After centrifugation, the supernatant was removed,

and 600 µL of 70% ethanol was added to the pellet and centrifuged at the conditions described above. The ethanol was aspirated, the pellet was air-dried, and the DNA rehydrated in 100 µL of DNA Rehydration Solution for 1 h at 65 °C.

Protocol 2 (kit with modification)

Genomic DNA was extracted using the commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) with a modification: the lysis step was carried out at a higher temperature (55 °C) and kept overnight, covering a 16 hours interval (adapted from Moré et al. 2011).

Protocol 3 (DNAzol)

Genomic DNA was extracted using a specific reagent (DNAzol Reagent, Invitrogen, USA) according to manufacturer´s instructions. Briefly, 1 ml DNAzol was added to 10 cysts solution or 50 mg of myocardium sample previously minced using a scalpel, followed by homogenization and centrifugation (5 min at 10,000 × *g* at 4 °C). Each resulting viscous supernatant was transferred to a new tube. Genomic DNA precipitation was carried out by adding 0.5 ml of cold 100% ethanol, homogenized, incubated for 3 min at room temperature and centrifuged at the conditions described above. Finally, genomic DNA was washed with 1 ml of cold 75% ethanol and centrifuged (same conditions described above), and the supernatant was discarded and the resulting pellet suspended in 100 µL of MilliQ water.

Protocol 4 (CTAB)

The pools of ten cysts and myocardium samples were incubated for 10 min with 100 µL of lysis buffer (lysozyme 10 mg/ml, SDS 10% and proteinase K 10 mg/ml) at 37 °C. This was followed by addition of 100 µL cetyl-trimethyl ammonium bromide (CTAB) and 100 µL of 5 M NaCl into the solution and incubation at 65 °C for 10 min. Finally, genomic DNA was isolated from the lysate by the phenol-chloroform method (Sambrook and Russel 2001), precipitated by cold ethanol, and solubilized in MilliQ water.

The concentration and quality of DNA extracted from each sample was analyzed using spectrophotometer NanoDrop 1000 (absorbance of 260/280 nm for purity evaluation) (Thermo Scientific, USA). After that, the DNA samples were stored at -20°C until use.

PCR and electrophoretic analysis

DNA extracted by the different protocols described above was submitted to the polymerase chain reaction (PCR) under the same conditions, using a set of primers (Sarco forward 5′-CGCAAATTACCCAATCCTGA-3′ and Sarco reverse 5′-ATTTCTCATAAGGTGCAGGAG-3′; Moré et al. 2011) for amplification of a 700 bp fragment (Figure 1) from the 18S rDNA gene. Each PCR was performed in a total volume of

25 µL, containing 10X buffer (Promega, USA); 10 mM dNTPs (Ludwig Biotec, Brazil); 50 µm of each primer (Sigma-Aldrich, Brazil); 1U Taq DNA polymerase (Promega, USA); and 50 ng of DNA as template. Genomic DNA extracted from a pool of 50 cysts of *Sarcocystis* spp. was used as positive control and MilliQ water was used as negative control. The PCR was carried out using a T100 thermal cycler (Bio-Rad, Singapore) under the following conditions: 5 min at 94 °C for the initial hot denaturation step, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 45 s at 72 °C, and a final extension step of 5 min at 72 °C. The PCR products were visualized by UV illumination after electrophoresis at 1% agarose gel stained with Gel Red Nucleic Acid Stain (Biotium, USA).

Statistical Analysis

The frequency of positive samples detected at each protocol was compared by the Chi-square test at a confidence level of 99%, using the SAS software version 9.4. This approach was used to evaluate the efficacy of the DNA extraction protocols on both the harvested cyst pools in PBS (Figure 2) or myocardium samples (Figure 3).

The probability of *Sarcocystis* spp. DNA detection was calculated with respect to the collection of one, two, or three tissue samples (simplicate, duplicate, and triplicate) per heart. Although there was uncertainty on the presence of cysts in all tissue samples because of their uneven spreading throughout the tissues, the molecular detection of the parasite was considered as "true positive" for the purpose of diagnosis. This was because all the hearts used had the *Sarcocystis* spp. infection confirmed by microscopic fresh examination.

RESULTS AND DISCUSSION

The kit with modification (protocol 2) showed better results with regard to DNA extraction from pools of ten cysts in PBS (Figure 2), with the *Sarcocystis* spp. DNA detected from 92.6% of the samples. This frequency was higher ($p=0.0003$) than the 48.1% (13/27) detection obtained using both the kit (protocol 1) and CTAB (protocol 4). DNAzol (protocol 3) showed a lower detection frequency (7.4%; 2/27) in comparison with kit with modification ($p<0.0001$), kit ($p=0.0008$), or CTAB ($p=0.0008$).

DNAzol was not appropriate for DNA extraction from pools of ten cysts in PBS due to the low levels of *Sarcocystis* spp. DNA detection. Conversely, DNAzol protocol showed better results for myocardium samples among the tested protocols (Figure 3). While the parasite DNA was detected in 48.1% (13/27) of the myocardium samples submitted to DNAzol extraction, lower frequencies of positive diagnosis were obtained using kit with modification (7.4%; 2/27; $p=0.0008$), kit (3.7%; 1/27; $p=0.0002$), and CTAB (0/27; $p<0.0001$).

No significant difference was found among CTAB, kit, and kit with modification. Furthermore, none of these methods could be recommended for bovine myocardium samples,

because the PCR was not effective for *Sarcocystis* spp. DNA detection after these three different extraction protocols.

Spectrophotometer analysis of the pools of 10 cysts revealed DNA concentration of 50 ng/µL and purity around 2.0 for both kit and kit with modification; and 200 ng/µL from DNAzol (purity of 2.0) and CTAB (purity of 1.7). Although kit with modification showed better PCR results, DNAzol (protocol 3) showed better purity and concentration evaluations of DNA isolated from the pools of 10 cysts. However, this protocol probably could not disrupt the cysts' walls satisfactorily. In turn, from the myocardium samples, the DNA concentration and quality obtained were of approximately 250 ng/µL and 1.8, respectively, for both kit and kit with modification; 200 ng/µL and 2.0 from DNAzol; and 200-1000 ng/µL and 1.8 from CTAB. Although all of the four DNA extraction protocols showed satisfactory values for DNA concentration and purity, it is important to highlight that the relative quantity of genomic DNA from the host tissues greatly overcomes the quantity of *Sarcocystis* spp. DNA isolated in myocardium samples.

PCR results of samples extracted with DNAzol were used to calculate the probability of a true positive molecular diagnostic of *Sarcocystis* spp. infection in cattle hearts, comparing the use of simplicate, duplicate or triplicate myocardium samples (Table 3). Samples extracted with DNAzol were chosen because this protocol resulted in higher frequency of detection of *Sarcocystis* DNA by PCR from myocardium samples. Frequency of *Sarcocystis* spp. DNA detection increased from 55.6% (5/9), when only the first myocardium sample collected from each heart was evaluated, to 77.8% (7/9), when both first and second samples of each heart were considered. Thus, re-sampling led to an increase of 22.2% (2/9) in molecular detection of *Sarcocystis* spp. in infected cattle hearts. However, no positive sample was found in two of the nine hearts evaluated (22.2%), even when the third myocardium sample was tested. Therefore, the evaluation of the third sample from each heart did not result in any increase in frequency of *Sarcocystis* spp. detection. Maximum frequency of true positive diagnosis of *Sarcocystis* spp. by DNA detection with multiple sampling (77.8%) was not satisfactory, considering the identification of 100% of infection reached by fresh examination of the hearts. Additionally to the uneven distribution of *Sarcocystis* cysts in the myocardium, their walls could be not completely lysed, making it difficult to extract DNA before amplification by PCR. Furthermore, myocardium samples contain large amounts of host proteins such as hemoglobin, as well as lipids which complicate DNA isolation and can also inhibit PCR (Babaei et al. 2011; Dubey and Lindsay 2006; Nantavisai et al. 2007).

There are several options for DNA extraction methods currently available. These methods include traditional protocols, such as those based on phenol-chloroform or CTAB, as well as a variety of safer and user-friendly commercial kits for DNA isolation. However, the choice of the best protocol depends mainly on the nature of the sample (Wu et al. 1995; Zhao et al. 2011). Furthermore, in some cases it is necessary to add modifications to the conventional protocols and/or pre-treatment steps on the samples, in order to improve the final DNA quantity and quality. These processes can include modifications of the incubation time and/or temperature, freeze-thaw cycles, glass beads, or lysis enzymes (Babaei et al. 2011; Moré et al. 2011; Rotureau et al. 2005; Zhao et al. 2011).

Comparing protocols 1 and 2 for the pools of ten cysts showed that simple modifications to the lysis step of the DNA extraction kit such as increasing incubation time and temperature, can improve the final PCR results. This improvement could be attributed to the effect of modification on the rupture of cysts, allowing the release of more genomic DNA (Babaei et al. 2011). On the other hand, although Moré et al. (2011) recommended the same commercial kit with modification for isolation of *Sarcocystis* DNA from tissue samples of bovine loin, this method was not effective in case of the bovine myocardium samples tested in the present study (Figure 3).

DNAzol is a commercial reagent largely used for total genomic DNA isolation from animal tissues (Chomczynski et al. 1997). The lower frequency of PCR positive samples obtained after DNAzol extraction on the pools of ten cysts (Table 1 and Figure 2) could be indicative of a low performance of this method in disrupting the cysts. However, DNAzol protocol resulted in higher frequency of *Sarcocystis* spp. DNA detection (48.1%) from myocardium samples (Table 2 and Figure 3), showing that this protocol provided efficient extraction of DNA from cysts in these samples.

Prevalence of *Sarcocystis* spp. in cattle is close to 100% in most regions around the world (Vangeel, et al. 2013), and the cardiac muscle is one of the privileged sites for chronic infection by this parasite (Dubey et al. 1989). However, it is possible that a smaller number or even no cyst is present specifically at the tissue site sampled. Thus, this can result in a false negative diagnosis for sarcocystosis because of the lack of *Sarcocystis* spp. DNA in the tissue sample, as well as due to the inefficacy of the DNA extraction protocol. Furthermore, as shown in this study, evaluation of multiple samples is strongly recommended to diminish the frequency of false negative diagnosis of *Sarcocystis* spp. in infected hearts. In addition to the use of multiple sampling and an adequate DNA extraction protocol, a fresh examination is needed to guarantee an accurate diagnosis of the presence or absence of tissue cysts of *Sarcocystis* spp. in cattle hearts. The choice of appropriate DNA extraction protocol depends on the sample characteristics, and this is essential to improve DNA isolation for PCR or other molecular analysis.

CONCLUSION

Microscopic examination allowed the identification of all bovine hearts infected by tissue cysts. DNA extraction methods had a significant influence on molecular diagnosis of *Sarcocystis* spp. in bovine myocardium, as well as on parasite DNA detection from pools of cysts. Among the tested protocols, kit with modification was most suitable for DNA extraction from cysts in PBS, while DNAzol reagent resulted in higher detection frequencies of *Sarcocystis* spp. DNA from bovine myocardium. Multiple sampling improved molecular diagnosis of *Sarcocystis* spp. in cattle heart.

Conflict of interest: The authors declare that they have no conflict of interest.

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Table 1 – Frequencies of *Sarcocystis* spp. DNA detection by PCR using four different DNA extraction protocols on pools of ten cysts in PBS

Cattle hearts	Three samples from each heart	Protocol 1. Kit	Protocol 2. Kit with modification	Protocol 3. DNAzol	Protocol 4. CTAB
		[a]Positive (+) or negative (-) result of PCR in polls of 10 cysts submitted to each DNA extraction protocol			
Heart 1	1.1	+	+	-	+
	1.2	+	+	-	+
	1.3	+	+	-	+
Heart 2	2.1	+	+	-	-
	2.2	+	+	-	-
	2.3	+	+	-	+
Heart 3	3.1	-	+	-	-
	3.2	-	+	-	-
	3.3	+	+	-	+
Heart 4	4.1	-	+	-	-
	4.2	+	+	-	+
	4.3	-	+	-	-
Heart 5	5.1	+	+	-	-
	5.2	-	+	-	+
	5.3	-	+	-	-
Heart 6	6.1	-	+	+	+
	6.2	+	+	-	-
	6.3	-	+	+	-
Heart 7	7.1	+	-	-	+
	7.2	+	+	-	+
	7.3	-	+	-	-
Heart 8	8.1	-	+	-	-
	8.2	-	+	-	+
	8.3	+	-	-	+
Heart 9	9.1	-	+	-	-
	9.2	-	+	-	-
	9.3	-	+	-	+
Total (positive samples)		13	25	02	13

[a] PCR positive sample shows a successful molecular diagnostic, including the DNA extraction step.

Table 2 – Frequencies of *Sarcocystis* spp. DNA detection by PCR using four different DNA extraction protocols on bovine myocardium samples

Cattle hearts	Myocardium sections (200mg)	Protocol 1. Kit	Protocol 2. Kit with modification	Protocol 3. DNAzol	Protocol 4. CTAB
		[a]Positive (+) or negative (-) result of PCR in myocardium subsections (50 mg)			
Heart 1	1.1	-	+	+	-
	1.2	-	-	-	-
	1.3	-	-	+	-
Heart 2	2.1	-	-	+	-
	2.2	-	-	+	-
	2.3	-	-	-	-
Heart 3	3.1	-	-	-	-
	3.2	-	-	-	-
	3.3	-	-	-	-
Heart 4	4.1	-	-	-	-
	4.2	-	-	+	-
	4.3	-	-	-	-
Heart 5	5.1	-	-	+	-
	5.2	-	-	+	-
	5.3	-	-	+	-
Heart 6	6.1	-	-	+	-
	6.2	-	-	+	-
	6.3	-	-	-	-
Heart 7	7.1	-	-	-	-
	7.2	-	-	+	-
	7.3	-	-	-	-
Heart 8	8.1	-	-	-	-
	8.2	-	-	-	-
	8.3	+	-	-	-
Heart 9	9.1	-	-	+	-
	9.2	-	-	+	-
	9.3	-	+	-	-
Total (positive samples)		01	02	13	0

[a] PCR positive sample shows a successful molecular diagnostic, including the DNA extraction step.

Table 3 – Probability of true positive PCR diagnostic of *Sarcocystis* spp. infection in cattle hearts considering samples extracted with DNAzol, and comparing the use of one, two, or three myocardium samples from each heart

Cattle hearts	**[a]Positive (+) or negative (-) diagnostic based on first, second and third samples analysis**			**Total of positive samples at each heart**
	1st	**1st and 2nd**	**1st and 2nd and 3rd**	
1	+	+ and -	+ and - and +	66.7%
2	+	+ and +	+ and + and -	66.7%
3	-	- and -	- and - and -	0.0%
4	-	- and +	- and + and -	33.3%
5	+	+ and +	+ and + and +	100%
6	+	+ and +	+ and + and -	66.7%
7	-	- and +	- and + and -	33.3%
8	-	- and -	- and - and -	0.0%
9	+	+ and +	+ and + and -	66.7%
Probability of true positive diagnostic	55.6% (5/9)	77.8% (7/9)	77.8% (7/9)	Mean 48.1%

[a]PCR positive sample shows a successful molecular diagnostic, including the DNA extraction step.

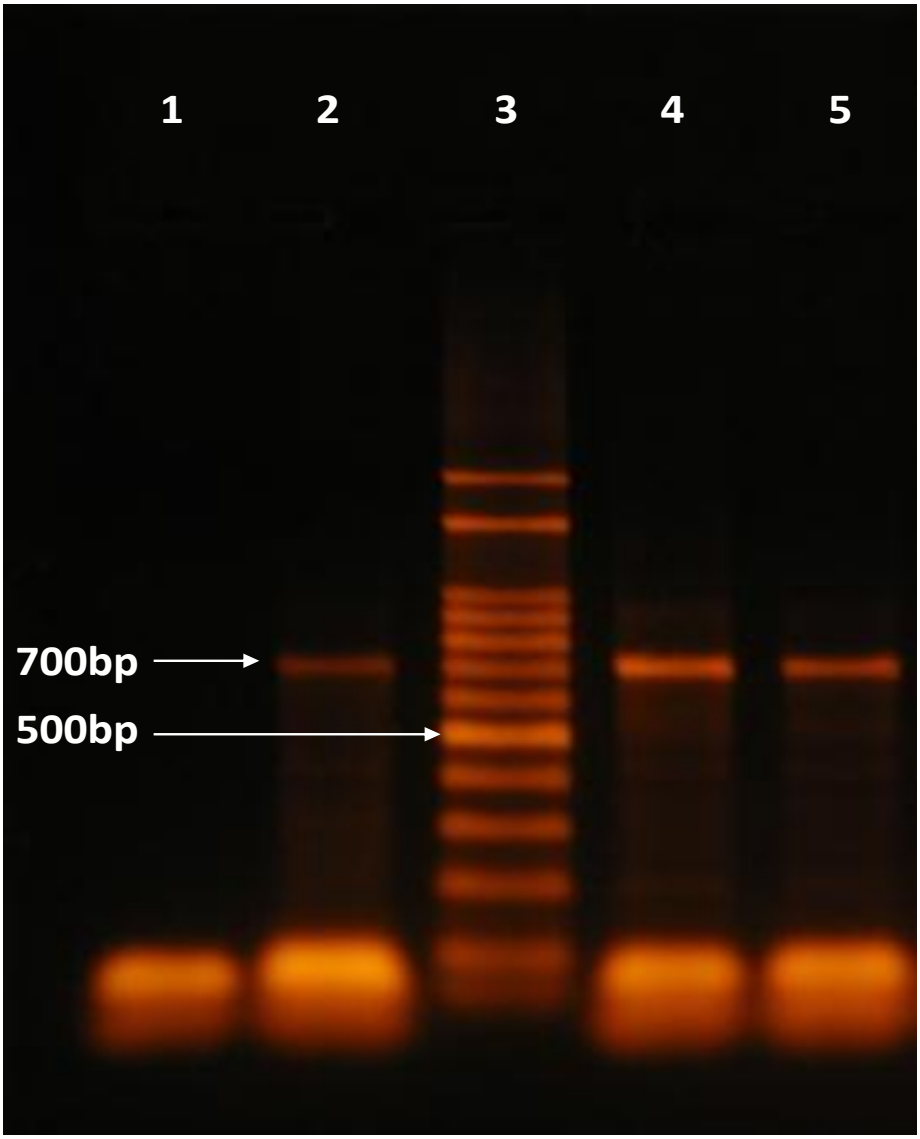

Figure 1 – Fragments of DNA identified by electrophoretic run after PCR performed for amplification of a fragment of the 18S rDNA gene (700 bp) from *Sarcocystis* spp. Line 1, negative control (MilliQ water); Line 2, positive control (*Sarcocystis* spp. DNA); Line 3, molecular marker (100-bp DNA ladder); Line 4, PCR amplification product obtained after DNA extraction from ten cysts in PBS using Protocol 2 (kit with modification); Line 5, PCR amplification product obtained after DNA extraction from bovine myocardium sample using Protocol 3 (DNAzol)

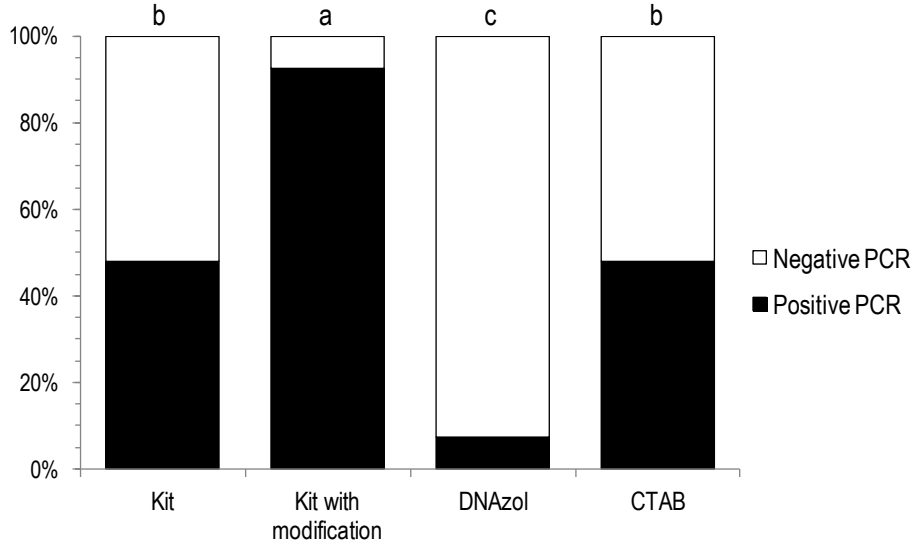

Figure 2 – *Sarcocystis* spp. DNA detection frequencies from polls of ten cysts in PBS using kit, kit with modification, DNAzol, or CTAB extraction protocols. Different lower-case letters above the columns indicate significant difference in the frequency of positive samples detected among protocols (Chi-square test at a 99% confidence level)

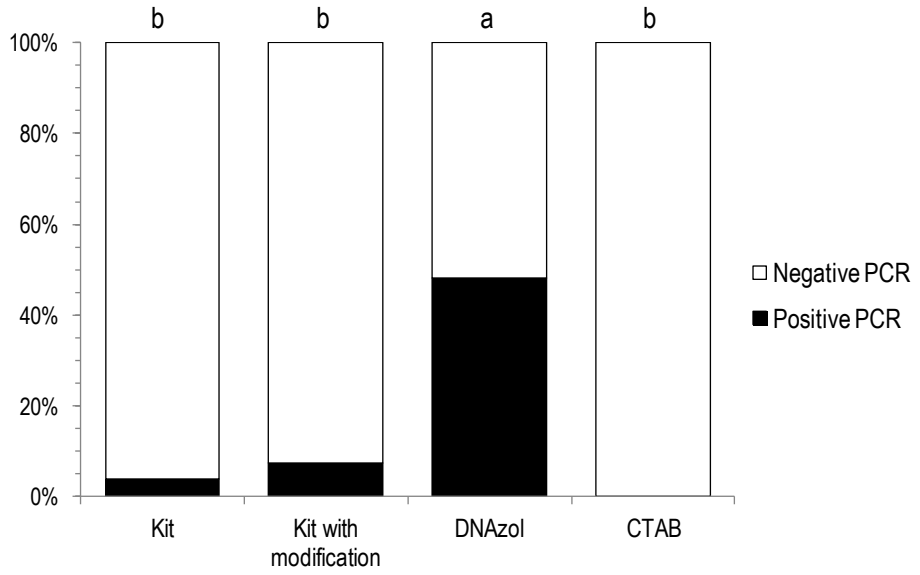

Figure 3 – *Sarcocystis* spp. DNA detection frequencies from bovine myocardium samples using kit, kit with modification, DNAzol or CTAB extraction protocols. Different lower-case letters above the columns indicate significant difference in the frequency of positive samples detected among protocols (Chi-square test at a 99% confidence level)